Characterisation of antimicrobial and heavy metal resistance among *Enterococcus* spp. from marine bivalve molluscs in Norway

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Table of Contents

ACKNOWLEDGEMENTS **7**

ABBREVIATIONS **10**

1.INTRODUCTION **13**

1.1 Aims of the study and study overview **13**

1.2 Genus *Enterococcus* **15**
1.2.1 Characteristics of the genus Enterococcus 15
1.2.2 Clades, kinship, and classification of Enterococcus 16

1.3 Habitats **20**
1.3.1 Enterococci in animals 20
1.3.2 Enterococci in the environment 21
1.3.3 Enterococci in the human body 22

1.4 Antimicrobial resistance **22**
1.4.1 The emergence of antimicrobial resistance 22
1.4.2 Antimicrobials: modes of action 25
1.4.3 Enterococci and antimicrobial resistance 25
1.4.4 Vancomycin resistant enterococci 29
1.4.5 Other antimicrobials 30

1.5 Heavy metal resistance **30**
1.5.1 Heavy metals in the environment 30
1.5.2 Link between heavy metal and antimicrobial resistance 31

1.6 How enterococci reach the ocean **32**
1.6.1 From anthropogenic sources to the marine environment 32
1.6.2 Bivalves as indicators of faecal pollution 33
1.6.3 Enterococci in seafood 34
1.6.4 Bivalves as vectors for the isolation of bacteria 34

1.7 Detection and identification **34**
1.7.1 Enumeration by the Most Probable Number method 34
1.7.2 Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry 35

2. MATERIALS & METHODS **36**

2.1 Study site, sampling, and samples **36**
2.1.1 Sampling of bivalve molluscs 36
2.1.2 Study site and time of year 37

2.2 Detection, enumeration, and verification **39**
2.2.1 Preparation of sample dilutions for the Most Probable Number (MPN) method 39
2.2.2 MPN method for detection and enumeration 40
2.2.3 Verification of enterococcal isolates on selective agar 41

2.3 Characterisation of isolates **42**
2.3.1 Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry 42

2.4 Antimicrobial susceptibility testing **42**
2.4.1 Screening for vancomycin resistance using agar selective methods 42
2.4.2 Antimicrobial susceptibility testing using the microdilution method 42

2.5 Heavy metal susceptibility testing **44**
2.5.1 Microdilution method 44
2.5.2 Agar dilution method 45
2.5.3 Minimum Inhibitory Concentration (MIC) for heavy metals and breakpoints 47

2.6 Molecular methods **49**
2.6.1 Extraction of DNA using DNeasy® Blood & Tissue Kit 49
2.6.2 Nanodrop and Qubit 49

2.7 Whole Genome Sequencing (WGS) **50**
2.7.1 Sequencing for studying antimicrobial resistance genes (Tromsø) 50
2.7.2 Bioinformatic analyses at K-res 50
2.7.3 Bioinformatic analyses at IMR 51

2.8 Culture collection **51**
2.8.1 Freezing isolates in pure culture for storage 51

2.9 Statistics and preparations of graphs **51**

3. RESULTS **52**

3.1 Sampling and samples **52**

3.2 Detection of presumptive enterococci in bivalve molluscs **52**
3.2.1 Prevalence and concentrations of enterococci 52
3.2.2 Faecal pollution in bivalve samples 54

3.3 Identification of ten enterococcal species among isolates **55**

3.4 Detection of antimicrobial and heavy metal resistant isolates **56**
3.4.1 Resistance to twelve antimicrobials was observed 56
3.4.2 Antimicrobial resistant enterococci were found in all counties 57

3.5 Heavy metal susceptibility testing **61**
3.5.1 Broth dilution method unsuccessful in testing for HMR 61
3.5.2 Agar dilution method successful for the detection of HMR in enterococci 62

3.6 Whole genome sequencing 64
3.6.1 WGS quality, phylogenetic analyses and detection of antimicrobial resistance genes 64
3.6.2 Detection of HMR genes 66

4. DISCUSSION 68

4.1 Prevalence of enterococci in marine bivalves 68

4.2 Effects of seasonality on the prevalence of enterococci 69

4.3 Antimicrobial and heavy metal resistance among bacterial isolates 69
4.3.1 Prevalence of antimicrobial resistant enterococci 69
4.3.2 Antimicrobial resistance genes 70
4.3.3 Heavy metal resistance 71
4.3.4 Co-selection of antimicrobial and heavy metal resistance 73

4.4 The origin of marine enterococci 73

4.5 Enterococci as indicators of faecal pollution 75

4.6 Methodological considerations 77

5. CONCLUSION 79

6. FURTHER STUDIES 80

7. REFERENCES 81

8. APPENDIX 94

8.1 MPN/100g for enterococci and *E. coli* samples 94

8.2 Enterococcal isolates and information 99

8.3 Recipes for growth media and other solutions 110

8.4 Distribution (n) of MIC values (mg/L) 120

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Abstract

Enterococci are one of today's leading causes of severe multidrug-resistant, hospital associated infections. Knowing that enterococci may enter the marine environment through sewage from the hospitals, community and run-offs from land, the fate of the enterococci and their prevalence in marine seafood organisms is poorly elucidated. This study examined 473 batch samples of marine bivalves collected along the coast of Norway in 2016, 2019 and 2020 of which 390 were blue mussel (*Mytilus edulis*), 44 European flat oyster (*Ostrea edulis*), 26 great scallop (*Pecten maximus*), four horse mussel (*Modiolus modiolus*), three ocean quahog (*Arctica islandica*), two pullet carpet shell (*Venerupis corrugata*), two sea urchin (*Strongylocentrotus droebachiensis*), and one each of cockle (*Cardiidae*) and pacific oyster (*Magallana gigas*). Quantification of enterococci was performed by a "Most Probable Number" (MPN) five times three tubes technique with FC Streptococcus broth and verification of each tube on *Enterococcus* agar. One typical colony was selected from all verified plates, allowing further study of the species variation within the same bivalve sample. Isolates were identified using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Enterococci were detected in 286 samples with concentrations ranging from <18 to 3500 MPN/100g, whereas the majority of the samples harboured low levels of enterococci. A total of 480 enterococcal isolates were retrieved from bivalves: 247 *Enterococcus faecium*, 115 *E. hirae* , 66 *E. faecalis*, 25 *E. durans,* seven *E. casseliflavus*, four *E. avium,* four *E. thailandicus*, three *E. gallinarum*, three *E. mundtii,* one *E. villorum* and five unidentified *Enterococcus* spp. All isolates were tested for antimicrobial and heavy metal resistance using culture-dependent broth and agar dilution methods. In total 392 of the 480 (82%) isolates were susceptible to all antimicrobial agents included in the study. Resistance towards one or more antimicrobials was observed in 88 isolates (18%), including 67 *E. faecium*, seven *E. faecalis*, three *E. casseliflavus*, three *E. avium*, three *E. gallinarum*, two *Enterococcus* spp., and one each of *E. durans*, *E. hirae* and *E. thailandicus*. The most seen resistance was against norfloxacin (10%) and ciprofloxacin (8%). Ampicillin, high-level gentamicin, quinupristin/dalfopristin and imipenem resistance were found in 1.2%, 0.8%, 1.7% and 4.5% of *E.* faecium, respectively. No resistance was seen towards vancomycin, linezolid, and nitrofurantoin. Different genes conferring resistance to antimicrobials and heavy metals, such as the aminoglycoside resistance-conferring gene *aac(6')-I,* were detected among the sequenced isolates. Eight isolates (2%) were resistant to copper, 37 (8%) were resistant to cadmium and presumptively none were resistant to zinc. Twelve isolates (3%) were resistant to both antimicrobials and heavy metals. Genetic analyses

of four *E. faecium* of which three expressed phenotypic resistance towards ampicillin, two to gentamicin and one to quinupristin/dalfopristin belonged to the hospital-associated sequence types (ST) 117 (n=2) and ST80 (n=1) and a novel sequence type 1484 (n=1). Core genome analyses found clustering between the two ST117 isolates. In conclusion, this study found low concentrations of enterococci in bivalves and only few resistant isolates. In isolates where resistance was found, genomic analyses linked them to the hospital environment, showing that enterococci found in bivalves can derive from the hospital environment.

Abbreviations

AM = Antimicrobial

AMR = Antimicrobial Resistance

AMOCLA = Amoxicillin/Clavulanic acid, a combination antimicrobial

APW = Alkaline Peptone Water

bp = Base Pair

CANS = Centre for New Antibacterial Strategies

cfu = Colony Forming Units

(cg) MLST = core genome Multi Locus Sequence Typing

CLSI = Clinical and Laboratory Standards Institute

CT = Cluster Type

DDD = Defined Daily Doses

ECDC = European Centre for Disease Prevention and Control

ECOFF = Epidemiological Cut-off Values

EUCAST = European Committee on Antimicrobial Susceptibility Testing

FIB = Faecal Indicator Bacteria

HAI = Hospital Acquired Infection

HCCA = α-cyano-4-Hydroxycinnamic Acid

HCl = Hydrogen Chloride

HGT = Horizontal Gene Transfer

HM = Heavy metal

HMR = Heavy Metal Resistance

IMR = Institute of Marine Research

K- Res = Norwegian National Advisory Unit on Detection of Antimicrobial Resistance, University Hospital of North Norway

MALDI-TOF MS = Matrix Assisted Laser Desorption Time Of Flight Mass Spectrometry

MHA = Mueller Hinton agar

MIC = Minimum Inhibitory Concentration

MLST = Multi Locus Sequence Typing

mM = Millimolar

MPN = Most Probable Number

MW = Molecular Weight

NaOH = Sodium Hydroxide

NFSA = Norwegian Food Safety Authority

NLHB = Norsk Legemiddelhåndbok

NMKL = Nordisk Metodikkomité for Næringsmidler

OUH = Oslo University Hospital

PCR = Polymerase Chain Reaction

Q/D = Quinupristin/dalfopristin, SYNERCID, a combination antimicrobial

ST = Sequence Type

STRAIN = a genetic variant in pure culture within a biological species

SYNERC = Synercid, Quinupristin/Dalfopristin, a combination antimicrobial

UiB = University of Bergen

UiT = The Arctic University of Norway

UiO = University of Oslo

VAN = Vancomycin

vanA = gene from *Enterococcus faecium* required for high-level resistance to glycopeptide antimicrobials

VKM = Norwegian Scientific Committee for Food Safety

VRE = Vancomycin Resistant Enterococci

WGS = Whole Genome Sequencing

WHO = World Health Organization

1.Introduction

1.1 Aims of the study and study overview

Given that little is known about the prevalence of enterococci in the marine environment in Norway, we sought to further elucidate this topic by examining enterococci from filter-feeding bivalves collected along the Norwegian coast. The following aims were defined:

1. Examine the prevalence of *Enterococcus* spp. in marine bivalve molluscs in Norway
2. Examine the effect of seasonality on the prevalence of enterococci
3. Characterise antimicrobial- and heavy metal resistance (HMR) patterns and associated genes in enterococci, and the possible co-selection of these traits
4. Examine the origin of the *Enterococcus* spp. isolates
5. Examine the rationale of applying enterococci as indicators of faecal pollution

To achieve this, the work also had to develop a method for heavy metal susceptibility testing of marine enterococci. The study consisted of isolation and identification of bacterial isolates, antimicrobial- and HMR testing and genetic analyses (Fig. 1.1).

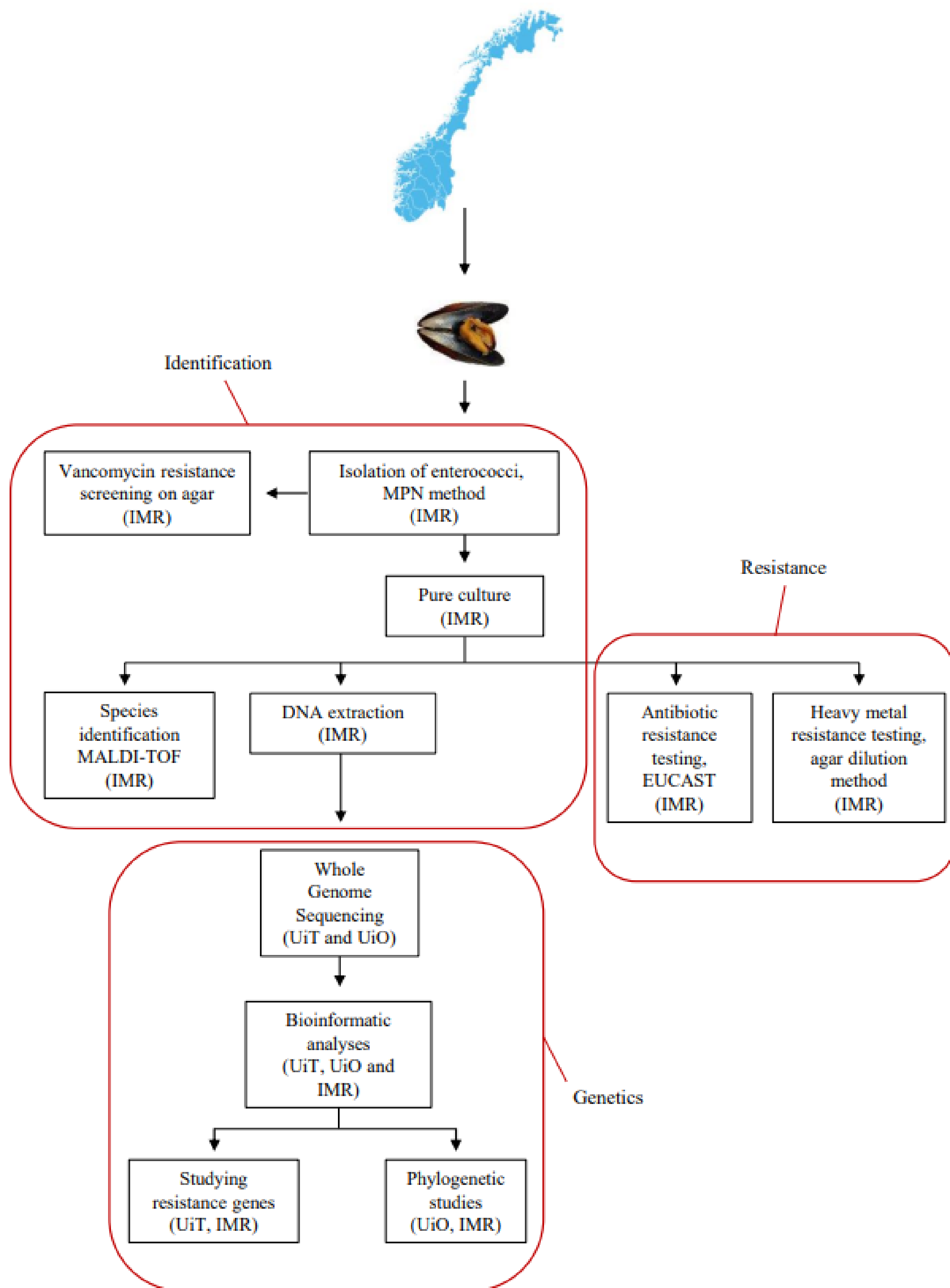

Figure 1.1: Study overview of the different steps throughout the project, starting with sampling, cultivation and characterisation of the bacterial isolates followed by antimicrobial and heavy metal susceptibility testing and DNA based methods. Identification and resistance testing were done at the IMR, and genetic studies were done at UiT, UiO and IMR. IMR: Institute of Marine Research; UiT: Arctic University of Norway; UiO: Oslo University Hospital.

1.2 Genus *Enterococcus*

1.2.1 Characteristics of the genus Enterococcus

Enterococci are gram-positive bacteria that belong to the genus *Enterococcus*. One of the aspects of enterococci that gives cause for great concern, is their intrinsic capability of becoming opportunistic pathogens, making them one of today's leading causes of severe multidrug-resistant, hospital associated infections (Clewell, 2014; Vignaroli et al., 2018). Enterococci were for the first time described in 1899, by the Frenchman M. E. Thiercelin. The same year, a similar organism was isolated and described from a lethal case of endocarditis by MacCallum and Hastings (Lebreton et al., 2014). The organism was later determined to have been *E. faecalis* and provided the first detailed insight into enterococci as a pathogen.

Enterococci naturally inhabit the gut of humans and animals (Agudelo Higuita & Huycke, 2014) but occur in smaller numbers than *Escherichia coli* (Duinker et al., 2020). Most *Enterococcus* species are commensal, harmless organisms, but a few are opportunistic human pathogens (Byappanahalli et al., 2012). Two of the most studied *Enterococcus* species, *E. faecalis* and *E. faecium*, are causing problems as etiological agents of nosocomial infections (Moellering, 1992; Morrison et al., 1997). *E. faecalis* is the most prevalent species in nosocomial infections while *E. faecium* has proven to be resistant to a large selection of antimicrobials (Morrison et al., 1997; Tendolkar et al., 2003). Previously, enterococci were considered as commensal organisms of little clinical importance (Hollenbeck & Rice, 2012), but since the mid-1980's, there has been a globally increasing occurrence of antimicrobial resistant enterococci, giving cause for concern (Bhattacharjee, 2016). The enterococci become resistant either through genetic mutation, recombination or horizontal gene transfer (Lebreton et al., 2013) after exposure to antimicrobials.

Common infections caused by enterococci, are urinary tract infections (UTIs), bacteraemia, endocarditis, neonatal infections, endodontic infections, abdominal and pelvis infections and in surgical wounds (Agudelo Higuita & Huycke, 2014; Gomes et al., 2006; Hidron et al., 2008; Moellering, 1992). Furthermore, enterococci are known for their ruggedness and unusual ability to adapt to varying environments, whether it being on inanimate surfaces or at sites of infection (Fiore, Elizabeth, Van Tyne, Daria, Gilmore, 2019), which makes them perfectly suited for surviving in a nosocomial environment. On the World Health Organization's list of

bacteria and pathogens for which new antimicrobials are urgently needed, vancomycin-resistant *Enterococcus faecium* is placed in the category of high priority (Asokan et al., 2019).

1.2.2 Clades, kinship, and classification of Enterococcus

Since the 1930s, enterococci were classified as group D streptococci (Sherman, 1937, 1938), belonging to the genus *Streptococcus*, but as of 1984, molecular analyses revealed that the species we today call *E. faecalis* and *E. faecium* were quite distantly related to other non-enterococcal streptococci (Schleifer, K. H., Kilpper-Balz, 1984) and the genus *Enterococcus* was established. *Enterococcus* belongs to the *Lactobacillales* order and the *Enterococcaceae* family, together with the other genuses *Bavariicoccus, Catellicoccus, Melissococcus, Pilibacter, Tetragenococcus* and *Vagococcus* (García-Solache & Rice, 2019). They are sphere-shaped, Gram-positive, non-spore forming bacteria, and often occur in pairs or chains (Hardie & Whiley, 1997; Murray, 1990). Being chemoorganotrophic (Byappanahalli et al., 2012; Ramsey et al., 2014), catalase negative (Hardie & Whiley, 1997; Klein, 2003; Murray, 1990) and obligatory homofermentative, they produce only lactic acid as a by-product upon fermenting glucose (Cornell University, 2009; Ramsey et al., 2014).

As of today, 60 valid species of *Enterococcus* species are officially known and classified into five groups (Table 1.1) based on their acid formation in sorbose broth and mannitol, and their ability to hydrolyse arginine (Agudelo Higuita & Huycke, 2014; Gilmore, 2002). This classification was suggested by Facklam and collaborators (Gilmore, 2002) , but one should keep in mind that this classification does not reflect the evolutionary relationships between the different *Enterococcus* species (Agudelo Higuita & Huycke, 2014; Gilmore, 2002) which would necessitate further analysis using 16S rRNA sequence relationships to provide different insights and suggest different groups of classification than those suggested by Facklam et al.

Table 1.1 A selection of 38 different species of the genus *Enterococcus* and the habitats where they currently are known to be found (Agudelo Higuita & Huycke, 2014; Byappanahalli et al., 2012)**.** Species marked in blue were isolated from bivalves in this study, as further described in the "Results" section.

Group	Species	Known habitat(s)	Human pathogen	References
1: Form acid in sorbose and mannitol, do not hydrolyse arginine (Gilmore, 2002)	*E. avium*	Human, animals	Yes	(D. Collins & Farrow, 1984; Cox & Gilmore, 2007; Ghosh et al., 2012; Nowlan, Sandra S., 1967)
	E. pseudoavium	Human		(Collins MD, Facklam RR, Farrow JA, 2020)
	E. malodoratus	Animal (cattle)		(D. Collins & Farrow, 1984)
	E. raffinosus	Human	Yes	(Collins MD, Facklam RR, Farrow JA, 2020; Murray, 1990)
	E. gilvus	Human		(Tyrrell et al., 2002)
	E. pallens	Human		(Tyrrell et al., 2002)
	E. devriesei	Animal (cattle)		(Švec, Vancanneyt, Koort, et al., 2005)
	E. phoeniculicola	Animal (bird)		(Agudelo Higuita & Huycke, 2014; Law-Brown & Meyers, 2003)
	E. canis	Animal (dog)		(Agudelo Higuita & Huycke, 2014; Naser et al., 2005)
	E. saccharolyticus	Animal (cattle), sewage		(Agudelo Higuita & Huycke, 2014; Layton et al., 2010; Rodrigues & Collins, 1990)
2: Form acid in mannitol but not in sorbose, hydrolyse arginine (Gilmore, 2002)	*E. faecium*	Human, animals, plants, insects	Yes	(Agudelo Higuita & Huycke, 2014; Cox & Gilmore, 2007; Klein, 2003; Layton et al., 2010; Schleifer, K. H., Kilpper-Balz, 1984; Ulrich et al., 2001)
	E. faecalis	Human, animals, plants, insects	Yes	(Agudelo Higuita & Huycke, 2014; Cox & Gilmore, 2007; Layton et al., 2010; Schleifer, K. H., Kilpper-Balz, 1984; Ulrich et al., 2001)
	E. canintestini	Animal (donkey)		(Agudelo Higuita & Huycke, 2014; Vaux et al., 1998)
	E. lactis			(Agudelo Higuita & Huycke, 2014; Morandi et al., 2012)
	E. mundtii	Humans	Yes	(M. D. G. S. Carvalho et al., 2004)
	E. thailandicus	Humans, animal (cattle)		(Agudelo Higuita & Huycke, 2014; M. D. G. S. Carvalho et al., 2008; Shewmaker et al., 2011; Sukontasing et al., 2007)
	E. sanguinicola			(Agudelo Higuita & Huycke, 2014; M. D. G. S. Carvalho et al., 2008)

3: Unable to form acid in neither mannitol nor sorbose, but hydrolyse arginine (Gilmore, 2002)	*E. villorum*	Animals (pig)		(Agudelo Higuita & Huycke, 2014; Vancanneyt et al., 2001)
	E. durans	Human, animals, insects	Yes	(D. Collins & Farrow, 1984; Cox & Gilmore, 2007; Layton et al., 2010)
	E. dispar	Human		(M. D. Collins et al., 1991)
	E. hirae	Animals, plants		(Farrow & Collins, 1985; Layton et al., 2010; Ulrich et al., 2001)
	E. silesiacus	Drinking water		(Švec et al., 2006)
	E. rotai			(Sedláček et al., 2013)
	Variants of *E. faecalis* and *E. faecium*			(Gilmore, 2002)
4: Do not produce acid in mannitol and sorbose, do not hydrolyse arginine (Agudelo Higuita & Huycke, 2014)	*E. asini*			(Agudelo Higuita & Huycke, 2014)
	E. sulfureus	Plant		(Martinez-Murcia & Collins, 1991)
	E. cecorum	Animal (chickens)		(Devriese et al., 1983; Williams et al., 1989)
	E. aquamarinus	Seawater		(Švec, Vancanneyt, Devriese, et al., 2005)
	E. plantarum			(Agudelo Higuita & Huycke, 2014)
	E. caccae	Human		(Agudelo Higuita & Huycke, 2014; M. da G. S. Carvalho et al., 2006)
	E. termitis	Animal (termite)		(Švec et al., 2006)
5: Form acid in mannitol but not in sorbose, fail to hydrolyse arginine (Gilmore, 2002)	*E. columbae*	Animal (pigeon)		(Devriese, 1990)
	E. rivorum			(Agudelo Higuita & Huycke, 2014)
	E. hermaniensis	Animal (dog)		(Koort et al., 2004)
	E. camelliae	Plant		(Sukontasing et al., 2007)
	E. viikiensis	Animal (broiler plant)		(Rahkila et al., 2011)
	Variants of: *E. casseliflavus* *E. gallinarum* and *E. faecalis* that fail to hydrolyse arginine	Plants, soil, human, animals Human, animals, insects	Yes Yes	(D. Collins & Farrow, 1984; Layton et al., 2010; J Orvin Mundt & Graham, 1986; Murray, 1990) (D. Collins & Farrow, 1984; Cox & Gilmore, 2007; Layton et al., 2010)
6: Ungrouped	*E. ureilyticus*			(Sedláček et al., 2013)
	E. quebencensis	Well water		(Sistek et al., 2012)
	E. italicus	Animal (cattle)		(Fortina et al., 2004)
	E. ureasiticus	Well water		(Sistek et al., 2012)

The large genomic differences between clinical and commensal isolates has divided *E. faecium* isolates in separate clades, three of which are Clade B, Clade A1 and Clade A2 (Fig. 1.2) (Lebreton et al., 2013). Clade B contains commensal strains that are rarely related to disease whereas Clade A1 contains hospital associated strains that are known to cause infection (Lebreton et al., 2013). Clade A1 strains have 25% larger genomes than strains in Clade B – this increase in genome size is due to their acquisition of extra mobile genetic elements and genes coding for resistance and virulence, which is typical for strains that survive in the hospital environment over time (Lebreton et al., 2013). Genes coding for resistance to vancomycin are more common in Clade A1 than in Clade A2 which contains strains with very diverse origins (Lebreton et al., 2013).

A method called Multilocus Sequence Typing (MLST) that has been used the last decades for molecular strain typing can be used to distinguish clones within the clades. MLST is based on the genetic sequences of seven specific so-called “housekeeping genes” (Homan et al., 2002), where each isolate of a given species will be characterised through the identification of alleles in the loci of these seven housekeeping genes (Homan et al., 2002; PubMLST, 2020). The alleles at each of these seven loci define the sequence type (ST) of the isolate (Homan et al., 2002). STs are species specific, meaning that for instance *E. faecium* STs should only be compared to STs of other *E. faecium* isolates. Based on whole genome sequences core genome MLST (cgMLST) schemes that contain a large number of specific genes have been made for several bacterial species to further distinguish strains (De Been et al., 2015).

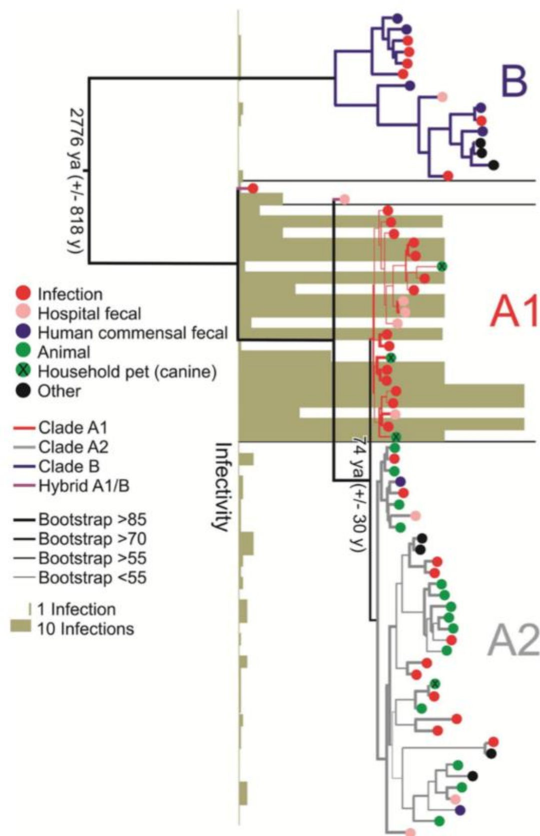

Figure 1.2: A Single Nucleotide Polymorphism (SNP) tree based on *E. faecium* core genomes showing the different clades B, A1 and A2 (Lebreton et al., 2013).

1.3 Habitats

1.3.1 Enterococci in animals

Enterococci are not only found in humans, but in mammals, amphibians, reptiles, and insects. In many mammal species, the colonisation of enterococci is age-dependent, for example will *E. faecalis*, *E. faecium* and *E. avium* dominate in preruminant calves but are then later replaced by *E. cecorum* (Lebreton et al., 2014). Terrestrial animal species that have been documented to contain enterococci include pig, cattle, chicken, donkey, sheep, goat, dog, cat, horse, wolf, lynx, bird, boar, partridge, red fox, wild rabbit and wild goose (Lebreton et al., 2014). Insects that have shown to contain enterococci include bees, flies, worms, beetles, termites and cockroaches (Lebreton et al., 2014), to mention a few.

Enterococci are also detected in marine animals (Lebreton et al., 2014). Linking the occurrence of enterococci to marine animals is of particular interest in this study as this could extend our understanding of the origin of marine enterococci. The common idea is that marine enterococci originate from faecal pollution from mammal species, which is very probable, but maybe there

are also specialised enterococci that have adapted to the marine environment over time. Enterococci have been registered in whale, dolphin, echinoderms, crustaceans, turtle, and different fish species.

1.3.2 Enterococci in the environment

Research has shown that enterococci can survive and multiply in many types of environments, including areas of no anthropogenic influence and without the presence of animal faeces (Byappanahalli et al., 2012). Examples of these environments include animal hosts (Lebreton et al., 2014), the gut of insects (Martin, J. D., Mundt, 1972), plants (Lebreton et al., 2014; J. O. Mundt, 1963), soil (Byappanahalli et al., 2012; Lassen et al., 2011; Lebreton et al., 2014; J. Orvin Mundt, 1961), marine sediments (Byappanahalli et al., 2012), recreational waters (Ator, L. L., Starzyk, 1976; Byappanahalli et al., 2012), aquatic and terrestrial vegetation (Byappanahalli et al., 2012), fermented foods and dairy products (Lebreton et al., 2014). They are known to be resilient bacteria and can survive for a longer period in areas of environmental stress and can survive in environments of fluctuating temperatures, which stands in contrast to the stable temperature of the gastrointestinal tract of warm-blooded animals (Byappanahalli et al., 2012). In many environmental habitats, strains of enterococci showing resistance to antimicrobials, have emerged, and been isolated with an alarming frequency the previous years. Thus, in scenarios where enterococci have become resistant and are causing harm to its host or the environment, it can be challenging to eliminate the enterococci with the antimicrobials and methods that are currently available (Bhattacharjee, 2016).

In the 1960s and 1970s, researchers found enterococci in the gastrointestinal tract (GI) tract and faeces of mammals (71,3%), reptiles (85,7%), birds (31,8%) and insects (53%) (Martin, J. D., Mundt, 1972; J. O. Mundt, 1963a). The fact that enterococci are inhabiting so many different environments, suggests that enterococci must have been members of gut microbiomes since the early Devonian period (~412 MYA), at least, and might be one of the earliest members of the GI tract microbiome (Agudelo Higuita & Huycke, 2014; Gilmore et al., 2013). The early Devonian period is the time when the last common ancestor of mammals, reptiles, birds and insects existed (Agudelo Higuita & Huycke, 2014; Selden, 2005).

1.3.3 Enterococci in the human body

Already from birth, humans are equipped with a microbiota that serves as a natural defence, keeping the human healthy and alive (Agudelo Higuita & Huycke, 2014). Especially the GI tract is vastly colonized by microbes, but also the skin, the oral cavity, the upper respiratory tract, and the vagina (Agudelo Higuita & Huycke, 2014). Genome analyses reveal that enterococci, having coexisted with the gut-environment of a host for millions of years, have a selective advantage in their acquisition of several nutrients needed for growth and survival, instead of carrying the genetic material otherwise needed for biosynthesis (Gilmore et al., 2013). Each site of the body, which is colonized by microbes, represents its own, specialized ecosystem that makes up a competitive environment that selects for the best adapted microbes (Agudelo Higuita & Huycke, 2014; Tannock, 1988). The bacterial diversity in these microbial communities is high; in the human colon, there are 10^{12} bacteria per gram of contents, of which enterococci constitute less than 1% (Agudelo Higuita & Huycke, 2014). The microbial communities in the intestine aid in tissue development and maintenance of the immune system homeostasis (Agudelo Higuita & Huycke, 2014; Berg, 1996). Enterococci being commensal bacteria, play an essential role in aiding in digestion and the metabolic pathways of the gut in healthy persons (Byappanahalli et al., 2012), while taking up nutrients mainly from the diet of their host or by cross-feeding with other microbes living in the same gut consortium (Gilmore et al., 2013). In a healthy individual, enterococci face many natural barriers preventing them from entirely colonizing the gut, such as the diversity of the intestinal microbiota and the low pH of the stomach (Agudelo Higuita & Huycke, 2014).

1.4 Antimicrobial resistance

1.4.1 The emergence of antimicrobial resistance

Bacterial resistance to antimicrobials is today considered one of the major challenges threating the global public health (WHO, 2015). Antimicrobial resistance is problematic because it reduces the effectiveness of antimicrobials used to treat infectious diseases in both humans and animals (NORM/NORM-VET, 2019). The discovery of antimicrobials began two centuries ago, and not much after the first antimicrobial resistant strains emerged. In 1928, almost by chance, the Scottish bacteriologist Alexander Fleming discovered that a *Penicillin* mould produced an antibacterial compound which he then named penicillin (Gaynes, 2017; Landecker, 2016). The discovery of penicillin and its curing effects led to much excitement among people at the time. Penicillin could be bought over-the-counter, requiring no

prescription, and was even added to certain cosmetic and skincare products (Bhattacharjee, 2016). With penicillin on the market and with increased knowledge about how to discover and mass produce antimicrobials, many other antimicrobials were discovered and added to the market in the 1940s and 1950s, such as vancomycin, chloramphenicol, erythromycin and streptomycin (Gaynes, 2017). Antimicrobials were viewed as wonder drugs, but unfortunately, the overuse and rapid dissemination of antimicrobials in large areas of the world, paved way for a new problem to arise: antimicrobial resistant bacteria (Bhattacharjee, 2016).

Works by Cassini et al. (Cassini et al., 2019) estimated that in 2015 in Europe, 670 000 persons were infected by antimicrobial resistant bacteria, of which 63% were related to the health care sector, and approximately 30 000 resulted in death. The burden was highest in Italy and Greece (Fig. 1.3) and resistance to third-generation cephalosporin-resistant *E. coli* was the most common infectant in Europe (Cassini et al., 2019). Only 69 deaths in Norway caused by antimicrobial resistant bacteria was reported that year which was very low compared to other European countries (Cassini et al., 2019).

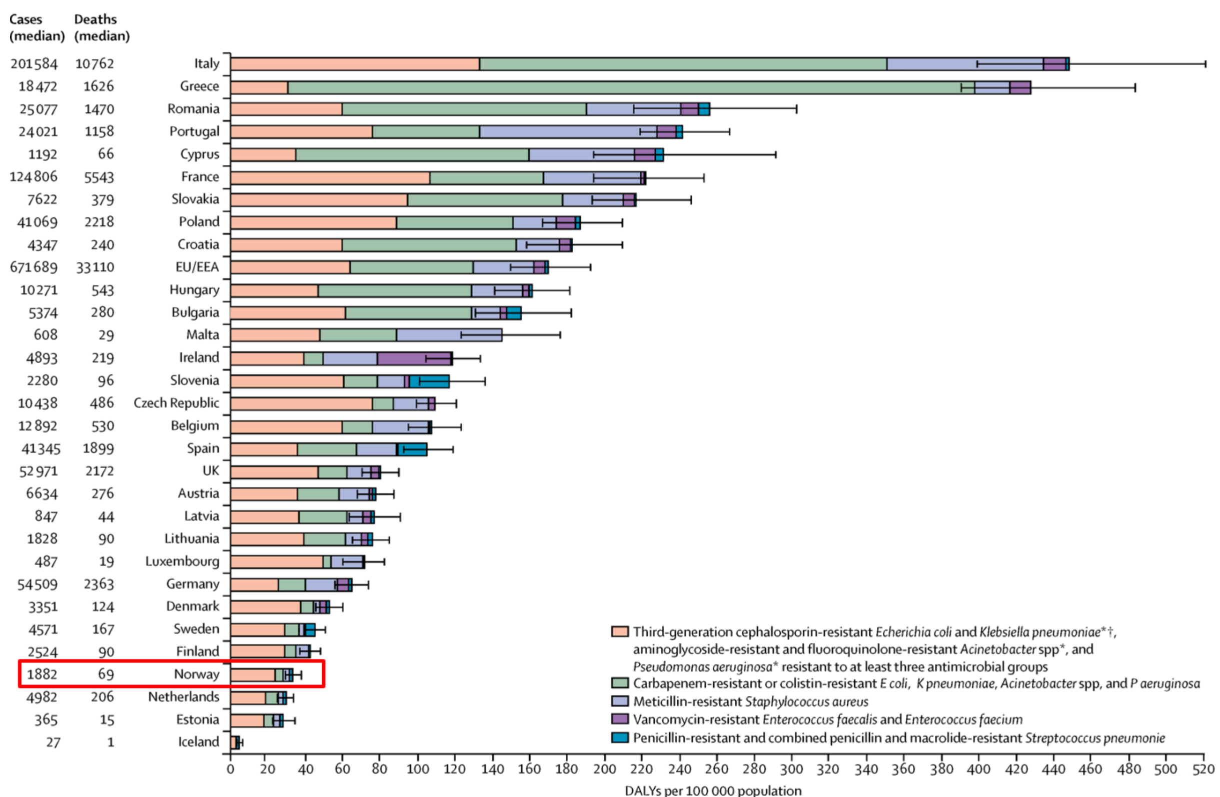

Figure 1.3: The burden of infections with antimicrobial resistant bacteria in DALYs (disability-adjusted life-years) in EU and the European Economic Area based on reported numbers to the European Antimicrobial Resistance Surveillance Network in 2015 (Cassini et al., 2019). Infections caused by vancomycin-resistant *E. faecalis* and *E. faecium* are indicated as dark purple and Norway is highlighted in red on the ranking.

A microorganism is said to be multidrug resistant when it is resistant to three or more of the six antimicrobial classes (see *1.4.2 Antimicrobials: modes of action*) (Bhattacharjee, 2016). Antimicrobial resistance in bacteria can be categorized as either intrinsic resistance, acquired resistance or tolerance (Kristich et al., 2014). Intrinsic resistance is when bacteria are naturally resistant to a given antimicrobial, without having been exposed to the antimicrobial before (Bhattacharjee, 2016). An example is gram-negative bacteria who are intrinsically resistant to vancomycin because the molecule is too large to cross their outer membrane (Bhattacharjee, 2016). Acquired resistance occurs when in a population of antimicrobial sensitive bacteria, some of the bacterial cells present become resistant when exposed to an antimicrobial.

The development of acquired resistance takes place either by point mutations or by the acquisition of resistance genes through horizontal gene transfer (HGT) (Bhattacharjee, 2016). HGT is when genetic material is transferred not in the "classical" way from a parent to its progeny generation, but from one cell to another already existing cell from the same generation (University of Leicester, 2020). HGT happens either by transformation, conjugation, or transduction (University of Leicester, 2020). Transformation happens when bacteria take up "available" DNA from their surroundings, commonly from dead bacteria that have lysed and released all their genetic content (University of Leicester, 2020). Conjugation is the direct transfer of genetic material from one cell to another by physical cell-to-cell contact, often involving two plasmids (University of Leicester, 2020). Transduction is the transfer of DNA from one cell to another via a type of viruses called bacteriophages, that specifically infect bacteria (University of Leicester, 2020). Bacteriophages are unique in the sense that they are incapable of replicating their own genomes and depend on their host organism to do this for them (University of Leicester, 2020). If the host cells keep replicating the bacteriophage genome, the host cells will eventually burst from all the newly formed virus particles in a process called the lytic cycle. Another form of transductional HGT is characterised by bacteriophages that can switch from the lysis cycle and enter a lysogeny state where they fuse their genome with the bacterial host chromosome and stay latent for several generations before they release themselves from the host chromosome (University of Leicester, 2020). In the stage where the lysogenic bacteriophage releases itself from the host chromosome, small sequences of the bacterial DNA is released with it before the phage moves on and infects a new bacterial host (University of Leicester, 2020). In these ways, bacterial DNA is spread horizontally.

1.4.2 Antimicrobials: modes of action

A common way to classify antimicrobials, is by dividing them based on which target they interact with in the microbial cell in order to cause growth inhibition. There are six major classes of antimicrobials: 1) antimicrobials that inhibit cell wall synthesis, 2) antimicrobials that disrupt the cell membrane, 3) antimicrobials that inhibit the synthesis of important metabolites, 4) antimicrobials that inhibit DNA synthesis (replication), 5) antimicrobials that inhibit RNA synthesis (transcription) and 6) antimicrobials that inhibit protein synthesis (translation) (Bhattacharjee, 2016). Another way to classify antimicrobials is according to how they affect the growth and survival of the bacteria. Antimicrobials that entirely kill the bacteria are called bactericidal antimicrobials (for example penicillins) (Bhattacharjee, 2016). Those that stop the growth of the bacteria but without killing them, are called bacteriostatic antimicrobials (for example chloramphenicol) (Bhattacharjee, 2016). Enterococci are tolerant to the bactericidal activity of antimicrobials that target the cell wall, such as β-lactams and vancomycin, and the only way to overcome their tolerance is by combining a cell wall active antimicrobial with an aminoglycoside (Fiore, Elizabeth, Van Tyne, Daria, Gilmore, 2019; Kristich et al., 2014).

1.4.3 Enterococci and antimicrobial resistance

The most concerning present-day aspect of certain enterococcal species, is their acquired resistance to powerful and last-resort antimicrobials, notably the glycopeptide vancomycin and oxazolidinone linezolid (Sadowy, 2018) (Fig 1.4). Enterococci are extremely good at adapting to their environment, and one of the ways they do this is by acquiring resistance to antimicrobials, causing them to survive in the hospital environment (García-Solache & Rice, 2019). Enterococci are known to be very sturdy bacteria, and have shown to be resistant towards common disinfectants and antiseptics, desiccation, UV radiation and starvation (Fiore, Elizabeth, Van Tyne, Daria, Gilmore, 2019; Hartke et al., 1998; Lebreton et al., 2017; Maraccini et al., 2012).

Enterococci showing such high tolerance to many environmental stressors limits the selection of antimicrobials that can be used in treating enterococcal infections. Not yet scientifically confirmed but interesting nonetheless, is the idea that enterococci can adapt to such harsh, nutrient-poor environments by entering a viable but non-culturable state (Del Mar Lleo' et al., 1998; Figdor et al., 2003; Fiore, Elizabeth, Van Tyne, Daria, Gilmore, 2019; Heim et al., 2002).

In a study from the Netherlands, hospital adapted ampicillin-resistant strains of *E. faecium* survived in vitro for 5,5 years, which is an astonishing duration and stresses the importance of good hospital hygiene (Wagenvoort et al., 2015).

By acquiring mobile genetic elements from their environment, multidrug resistant strains arise and cause problems (Clewell, 2014; Vignaroli et al., 2018) such as prolonged hospitalisation time, additional treatment costs and increased risk of death (García-Solache & Rice, 2019). *E. faecalis* is the species most involved in hospital acquired infections (HAIs), and is more virulent than *E. faecium*, but infections caused by *E. faecium* are harder to treat due to several intrinsic resistance mechanisms and their increase in acquired antimicrobial resistance mechanisms (García-Solache & Rice, 2019). *E. faecalis* used to account for around 50% of all enterococcal HAIs, but the trend is turning and now *E. faecium*-caused infections are becoming more common due to the rise of vancomycin and β-lactam resistant *E. faecium* strains (García-Solache & Rice, 2019). *E. faecium* is much better than other species at acquiring resistance to ampicillin and vancomycin, which is why most vancomycin resistant enterococci are ampicillin-resistant *E. faecium* and are difficult to treat once infection with these occurs (de Kraker et al., 2013). The molecular mechanisms for developing vancomycin resistance are similar in *E. faecium* and *E. faecalis*, but vancomycin resistance appears more often in *E. faecium* (in 80% of isolates) than in *E. faecalis* (10% of isolates) (García-Solache & Rice, 2019).

Enterococci have until today proven capable of developing resistance towards all classes of antimicrobials that exist (Fiore, Elizabeth, Van Tyne, Daria, Gilmore, 2019). They are intrinsically resistant to more or less all cephalosporins and to clinically achievable concentrations of aminoglycosides (García-Solache & Rice, 2019; Kristich et al., 2014). They are also intrinsically susceptible to vancomycin, tetracyclines and erythromycin although acquired resistance to these antimicrobials is quite common (García-Solache & Rice, 2019). *E. gallinarum* and *E. casseliflavus* are known to be intrinsically low-level resistant to vancomycin (Bhattacharjee, 2016) due to expression of a ligase called *VanC* (NORM/NORM-VET, 2019), but fortunately, infections caused by these enterococcal species happen quite seldom (Gold, 2001) . The more recent antimicrobials linezolid, tedizolid and daptomycin are active against enterococci, and the streptogramin quinupristin/dalfopristin (Q/D) is only active against *E. faecium* (García-Solache & Rice, 2019). Resistance to fluoroquinolone antimicrobials is common in clinical *E. faecium* strains, but they still have an effect on other enterococcal species

(García-Solache & Rice, 2019). Ampicillin is usually the antimicrobial of first choice if the infectant strain is susceptible and the patient can tolerate ampicillin (García-Solache & Rice, 2019).

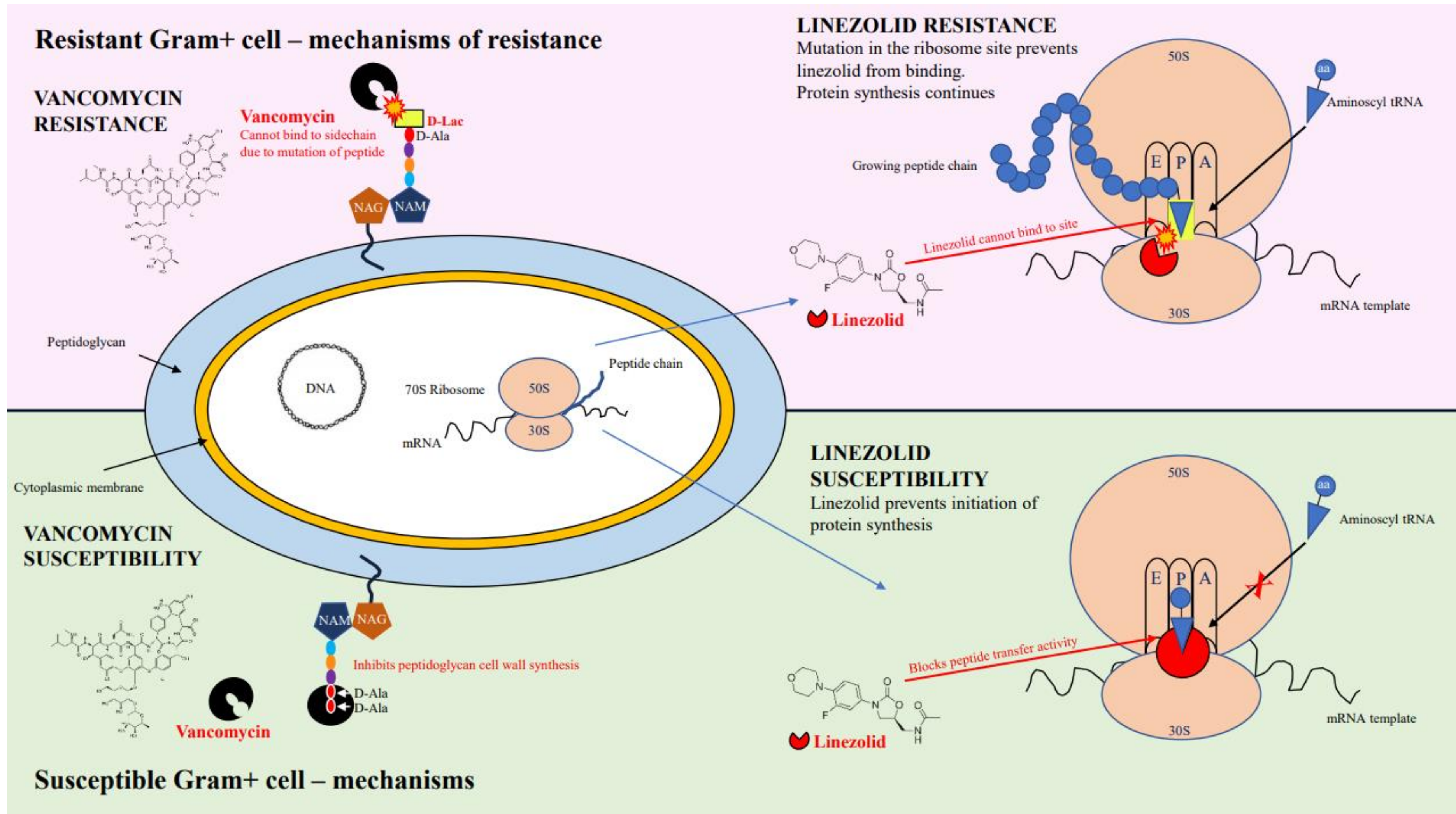

Figure 1.4. The different modes of action of vancomycin and linezolid. The upper section (pink) depicts a scenario where the gram-positive bacterium has acquired resistance towards the antimicrobial. The lower section (green) depicts a scenario where the gram-positive bacterium is susceptible to the antimicrobial. Figure: Amalie Heim.

1.4.4 Vancomycin resistant enterococci

Vancomycin is a glycopeptide antimicrobial which was first isolated from *Streptomyces orientalis* in the 1950s, and the name vancomycin derives from the word "vanquish" (Bhattacharjee, 2016). Vancomycin is considered a last resort antimicrobial, that should be used either if treatment with beta-lactams has failed or if the patient is allergic to beta-lactams (Bhattacharjee, 2016). It is effective in treating infections caused by gram positive bacteria that have become resistant to other antimicrobials, most importantly enterococci and Methicillin-resistant *Staphylococcus aureus* (MRSA). Gram negative bacteria are resistant to glycopeptide antimicrobials. In treating multidrug-resistant enterococci, vancomycin is one of very few antimicrobials that has shown to be efficient (Bhattacharjee, 2016). Enterococci are the third most common cause for HAIs in Europe (ECDC, 2013; Hegstad et al., 2020) and the fifth most common bacterial genus found in blood culture isolates in Norway (Hegstad et al., 2020; NORM/NORM-VET, 2019).

Cassini et al. (Cassini et al., 2019) found that in 2015, vancomycin-resistant *E. faecalis* and *E. faecium* represented the 8th most common cause of infections caused by antimicrobial resistant bacteria in Europe, and that the burden of this infection is highest in persons aged 65-69. Resistance to vancomycin is caused by certain gene clusters that contribute to changes in the peptide side chains that are important for the binding of vancomycin to the cell wall, these changes prevent vancomycin from binding to the bacterial cell wall making treatment with vancomycin inefficient (Courvalin, 2006; Hegstad et al., 2020). As of today, nine different gene clusters are known to cause vancomycin resistance in enterococci: *vanA*, *vanB*, *vanC*, *vanD*, *vanE*, *vanG*, *vanL*, *vanM* and *vanN*, where *vanA* and *vanB* are the most common acquired gene clusters on a worldwide basis (Hegstad et al., 2020). The gene cluster *vanC* is intrinsic to *E. casseliflavus* and *E. gallinarum*, whereas the other gene clusters are acquired by either *E. faecium* or *E. faecalis*, often in association with mobile genetic elements such as plasmids or integrative conjugative elements (Hegstad et al., 2020).

Vancomycin became the main form of treatment against aminoglycoside-resistant enterococci in the late 1970's and beginning of the 1980's (Fiore, Elizabeth, Van Tyne, Daria, Gilmore, 2019; Kirst et al., 1998), but in 1984, the first vancomycin resistant *Enterococcus* isolate emerged, first in Europe (Fiore, Elizabeth, Van Tyne, Daria, Gilmore, 2019; Leclercq et al., 1988; Uttley et al., 1988) and later in the US (Fiore, Elizabeth, Van Tyne, Daria, Gilmore, 2019; Sahm et al., 1989). Today, vancomycin resistant enterococci are spread across the world,

and in some hospitals up to 80% of all *E. faecium* isolates show resistance to vancomycin (Arias, C.A. and Murray, 2008; Fiore, Elizabeth, Van Tyne, Daria, Gilmore, 2019; Weiner et al., 2016). In Europe, the presence of vancomycin resistant *E. faecium* has been increasing since 2016, and in Norway the VRE levels have varied between 2010 and 2019 (Hegstad et al., 2020).

1.4.5 Other antimicrobials

The oxazolidinone linezolid is another last resort antimicrobial. In 2000, linezolid was approved by the FDA for clinical use. But already in 2001 (Fiore, Elizabeth, Van Tyne, Daria, Gilmore, 2019; Gonzales et al., 2001), the first linezolid-resistant strains emerged in the US, and in 2002 in the UK (Auckland et al., 2002; Fiore, Elizabeth, Van Tyne, Daria, Gilmore, 2019). The resistant isolates were sequenced, and a G2576U mutation, well-known to cause resistance, was found in the 23S ribosomal RNA subunit (Fiore, Elizabeth, Van Tyne, Daria, Gilmore, 2019; Marshall et al., 2002).

The glycylcycline tigecycline has also been used to combat vancomycin resistant enterococci (Fiore, Elizabeth, Van Tyne, Daria, Gilmore, 2019). In certain cases, resistance towards tigecycline itself occurs, but in most cases the treatment is successful, especially when combined with other antimicrobials (Fiore, Elizabeth, Van Tyne, Daria, Gilmore, 2019). Between 2007 and 2015, 73 *E. faecium* and *E. faecalis* isolates resistant to tigecycline, were collected in Germany (Fiedler et al., 2016; Fiore, Elizabeth, Van Tyne, Daria, Gilmore, 2019). The researchers found mutations in different efflux pumps that are linked to the development of tigecycline resistance (Fiedler et al., 2016; Fiore, Elizabeth, Van Tyne, Daria, Gilmore, 2019).

1.5 Heavy metal resistance

1.5.1 Heavy metals in the environment

Heavy metals (HMs) are natural elements that can be found in the planet’s crust and are typically characterised by a high atomic mass (Koller & Saleh, 2018) and a density of at least five times that of water (Tronsmo et al., 2016). Examples of HMs are copper, cadmium, lead, zinc, arsenic, nickel, mercury, selenium, and cobalt (Masindi & Muedi, 2018). Although naturally occurring, HMs have been spread in large quantities in nature due to anthropogenic activities and thus impacting the life of humans and marine and terrestrial organisms (Masindi

& Muedi, 2018). HMs are toxic to most life forms and don't degrade over time and thus bioaccumulate in the food chain (Masindi & Muedi, 2018).

Over the years, large quantities of heavy metals have been released into the ocean from effluent containing HMs that has been discharged from industries all over the world (Masindi & Muedi, 2018). This is concerning due to the negative impact this has one the marine flora, fauna, ecosystem – and for the development of heavy metal resistant bacteria. In bacteria, several copper resistance mechanisms have been described – some of which are intrinsic, others acquired after long-time exposure to environments with high concentrations of copper (Wastson et al., 2019).

In this study, the heavy metals copper, zinc and cadmium were selected for heavy metal susceptibility testing of the enterococcal isolates due to the high use of these as animal feed supplements in Norway (Tronsmo et al., 2016; Wastson et al., 2019). In 2016 in Norway, the total input of these metals to the Norwegian coastal areas was estimated to be 2 tonnes of cadmium, 576 tonnes of zinc and 1251 tonnes of copper (Weideborg et al., 2003), but these figures are probably an underestimation of the true numbers. The very high amount of copper input is due to fish farming because the fish cages are covered with chemicals containing copper to prevent algal growth (Weideborg et al., 2003).

1.5.2 Link between heavy metal and antimicrobial resistance

Inside all living cells, copper naturally plays an important role in enzymatic processes as an essential cofactor, but in too high concentrations, copper will become toxic to the cell especially due to its reactive nature (Hasman & Aarestrup, 2002). Therefore, to maintain a normal level of intracellular copper, all organisms have developed mechanisms for regulating copper homeostasis (Hasman & Aarestrup, 2002). In bacteria, the best studied copper homeostasis system is the *cop* operon from *Enterococcus hirae*, which consists of the four genes *copY*, *copZ*, *copA* and *copB* that are all controlled by the same promoter (Hasman & Aarestrup, 2002). The genes conferring copper resistance are usually located on plasmids and can thus be transferred to other bacteria (Hasman & Aarestrup, 2002). On the contrary, genes involved in regulating copper homeostasis are located on the chromosomes and not on the plasmids (Hasman & Aarestrup, 2002).

Hasman & Aarestrup (2002) detected a transferable gene called *tcrB* located on plasmids which was found to be responsible for mediating copper resistance in *E. faecium* isolated from pigs, the gene was strongly correlated to glycopeptide and macrolide resistance. In the same study,

they chose to look for copper resistance in pigs and other livestock animals due to the widespread use of copper, usually in the form of copper sulphate ($CuSO_4$), as a supplement in animal feed (Hasman & Aarestrup, 2002). Copper and other metals are added to animal feed due to the growth promoting effects of these elements (Wastson et al., 2019). The microbiota of animals that eat copper supplemented foods is therefore expected to contain bacteria that have adapted to higher copper concentrations and might thus become resistant to copper (Hasman & Aarestrup, 2002).

In Norway in 2012, the total amount of feed produced for pigs was approximately 469.000 tonnes, of which ten tonnes consisted of copper, and seven tonnes of zinc (Tronsmo et al., 2016; Wastson et al., 2019). For poultry, the total produced amount of feed was 428.000 tonnes, and copper and zinc represented eight and 48 tonnes of these, respectively (Tronsmo et al., 2016; Wastson et al., 2019). Zinc is added to the feed of pigs to prevent piglet diarrhoea, and it has been reported that pigs in Norway are exposed to twice the amount of zinc needed to fulfil their physiological needs (Norwegian Food Authority, 2014; Wastson et al., 2019). This indicates that pigs and poultry are exposed to much higher concentrations of heavy metals than what is required (Wastson et al., 2019) which in turn will affect the microbiota within these animals contributing to the development of heavy metal resistant bacteria.

1.6 How enterococci reach the ocean

1.6.1 From anthropogenic sources to the marine environment

As enterococci are, to our present-day knowledge, naturally deriving neither from the sea or the aquatic environment, any enterococcal bacteria found in marine samples is thought to originate from anthropogenic sources such as wastewater effluent (Vignaroli et al., 2018), urban sewage, sewage released from recreational boats, ship traffic, leaking septic systems, runoffs and domestic animal and wildlife waste (Boehm & Sassoubre, 2014).

On the other hand, some studies show that populations of enterococci found in marine sediments and soils may actually be endogenous to these environments and do not derive from faecal origins. Works by Whitman et al. illustrated that the macrophytic green algae *Cladophora* can serve as a great reservoir for the faecal indicator bacteria (FIB) *E. coli* and enterococci in both fresh and marine waters with bacterial densities surpassing 100 000 CFU/g dry weight algae (Byappanahalli et al., 2012; Whitman et al., 2003).

Moreover, enterococci are commonly used as faecal indicator bacteria (FIB) together with *E. coli* in for example seafood quality analyses. If enterococci can actually survive and thrive in the marine environment over time, without necessarily being transferred from land to sea, using enterococci as a FIB would not be useful (Byappanahalli et al., 2012; Byappanahalli & Fujioka, 2004; Desmarais et al., 2002). This would contradict the idea that enterococci are solely deriving from anthropogenic sources and shows the importance of performing genomic analyses of the bacterial isolates to look for differences between marine and clinical isolates and attempt to trace their origins.

1.6.2 Bivalves as indicators of faecal pollution

When examining the safety of bivalve molluscs for human consumption, screening for foodborne viruses is of particular importance (Duinker et al., 2020). The detection of either of the faecal indicator bacteria *E. coli* or enterococci in the sample indicates faecal contamination and thus increases the chances of finding human pathogenic viruses or other infectious agents (Duinker et al., 2020). One of the unique characteristics of the bacteria belonging to the genus *Enterococcus*, is their ability to grow in the presence of salt (6.5% NaCl) (Byappanahalli et al., 2012). Enterococci show a higher salt tolerance than *E. coli* and other faecal coliforms, which puts them in a better position for being indicators of human health risk in marine recreational waters (Byappanahalli et al., 2012).

The "perfect" faecal indication bacterium (FIB) is defined by its lack of virulence, being easy and fast to enumerate, its ability to survive in extra enteric environments similar to that of other pathogens and its presence is strongly related to the presence of pathogens (Byappanahalli et al., 2012; Cabelli et al., 1979). Since faeces of animals and humans are densely colonized by enterococci, enterococci are well suited for use as FIB in seafood analyses or in water quality analyses (Byappanahalli et al., 2012) of recreational waters and drinking water. The detection of *E. coli* indicates a more recent faecal contamination whereas the detection of enterococci can be an indication of faecal contamination that took place a while ago. Knowing that the longevity of *E. coli* outside of the intestinal tract is rather short, and that enterococci can survive in various environments for much longer time, additional screening for enterococci could strengthen the existing methods when looking for FIB in water, sediment, soil or seafood samples.

1.6.3 Enterococci in seafood

If enterococci can exist in marine environment, it also implies that other organisms living in the same environment can take up these bacteria, for example through water filtration as blue mussels do, or fish filtrating water through the gills. Whether the presence of enterococci in seafood can have a negative impact on the consumer, depends on the concentration of enterococci in the food and if the seafood has been prepared using high temperatures in cooking before eating. Getting sick from eating raw oysters is a classic example for illustrating what improper heating of seafood can lead to, as oysters can contain toxins produced by algae or bacteria. Many of these toxins are heat tolerant and require high temperatures to be broken down, and the same would apply if enterococci were present in the seafood – heating up to at least 80°C would ensure the elimination of enterococci (Martínez et al., 2003). A study in Tunisia found enterococci in different types of seafood: bream, clams, cuttlefish, mackerel, mullet, octopus, pandora, salmon, sardine, saupe, sea bass, shark, shrimp, sole, squid (Ben Said et al., 2017) – the source of contamination was not examined, and it was not found whether the presence of enterococci in the seafood was due to faecal-polluted waters or if enterococci were transferred during fish evisceration and human handling.

1.6.4 Bivalves as vectors for the isolation of bacteria

Bivalve molluscs, often largely represented by the blue mussel (*Mytilus edulis*), are well suited as vectors for the isolation of bacteria. An adult blue mussel with a shell length of 60 mm can filter from 12 to 240 litres of seawater daily (Cranford et al., 2011). Particles of 4 µM are retained with 100% efficiency (Møhlenberg & Riisgård, 1978), enterococci are 0.6-2.5 µM (HD, 2021) in comparison and are thus readily filtered by blue mussels and other bivalves.

1.7 Detection and identification

1.7.1 Enumeration by the Most Probable Number method

For the detection and enumeration of enterococci in bivalves, the Most Probable Number (MPN) method APHA 2001 for enterococci and faecal streptococci in foods (Hartman et al., 2001) was used. The MPN method is a standardized method used to estimate the number of bacteria per 100 grams sample material. The method is based on serial dilutions of the sample in tubes and observing positive tubes after incubation, often identified by a colour change in the medium (Hartman et al., 2001; Zimbro & Power, 2003). An MPN value based on the number of positive tubes is observed after incubation and gives an estimate on the actual number of bacteria per 100 grams of raw sample.

1.7.2 Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry

For the identification of the bacterial isolates, the Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) technology was used. MALDI-TOF uses mass spectrometry to measure the mass of proteins within the bacterial cell. Because bacteria have a unique protein composition, measuring the protein mass can distinguish between species in a process known as "peptide fingerprinting" (Tuma, 2003). The MALDI-TOF instrument contains an ionization source, an analyser, and a detector. Bacterial cell material is mixed with an organic matrix and protonated by ionization inside the MALDI-TOF instrument. The protonated sample molecules enter a gas phase and move into the analyser (Tuma, 2003). The time it takes for a molecule to reach the detector when moving through the analyser, is affected by its mass, hence the method name "Time of Flight" (Tuma, 2003). After detecting each molecule, the protein masses are presented as spectral peaks and compared to a large database containing already known spectra for bacterial species (Tuma, 2003). Each analysed isolate receives a score between 0 and 3, scores of 2 or higher indicate species identifications, a score between 1.7 and 1.9 indicates genus identification and a score below 1.7 indicates no identification (Schulthess et al., 2014). *E. casseliflavus* is known to often lie within the range of 1.7 to 1.9 and thus being more difficult to identify on species level.

2. Materials & Methods

2.1 Study site, sampling, and samples

2.1.1 Sampling of bivalve molluscs

Sampling was coordinated with the annually ongoing inspection programme by the Norwegian Food Safety Authority (NFSA), where bivalve molluscs are regularly collected from approved farming locations along the Norwegian coast and shipped to the IMR for analysing. Additional eleven samples were collected by IMR. Between November 13th, 2019 and July 28th, 2020, 228 samples were analysed, of which 192 were blue mussel (*Mytilus edulis*), 19 European flat oyster (*Ostrea edulis*), 12 great scallop (*Pecten maximus*), and one each of cockle (fam. *Cardiidae*), green sea urchin (*Strongylocentrotus droebachiensis*), ocean quahog (*Arctica islandica*), pacific oyster (*Magallana gigas*) and pullet carpet shell (*Venerupis corrugata*) (Fig. 2.1).

In addition, 245 frozen homogenates from 2016 from the same inspection programme by the NFSA were included. From these, 198 were from blue mussel (*Mytilus edulis*), 25 from European flat oyster (*Ostrea edulis*), 14 from great scallop (*Pecten maximus*), four from horse mussel (*Modiolus modiolus*), two from ocean quahog (*Arctica islandica*), one each from pullet carpet shell (*Venerupis corrugata*) and green sea urchin (*Strongylocentrotus droebachiensis*). In total, 475 samples from 2016, 2019 and 2020 were analysed in this project.

All samples were kept cold (at 4°C) and transported overnight to the microbiology lab. Analyses were commenced within 24 hours after sampling.

Figure 2.1: Samples of different bivalve mollusc species and green sea urchin were used in this study. A: Blue mussel (Eivind Senneset, IMR), B: European flat oyster (Øystein Paulsen, IMR), C: Great scallop (Ragni Olssøn, IMR), D: Cockle (Espen Bierud, IMR), E: Horse mussel (Erling Svensen, IMR), F: Ocean quahog (Erling Svensen, IMR), G: Pacific oyster (Torjan Bodvin, IMR), H: Pullet carpet shell (Erling Svensen, IMR), I: Green sea urchin (Erling Svensen, IMR).

2.1.2 Study site and time of year

All samples were collected along the coast of Norway (Fig. 2.2) from the counties Agder, Nordland, Rogaland, Troms & Finnmark, Trøndelag and Vestland. Samples were sampled during winter, spring, summer, and autumn.

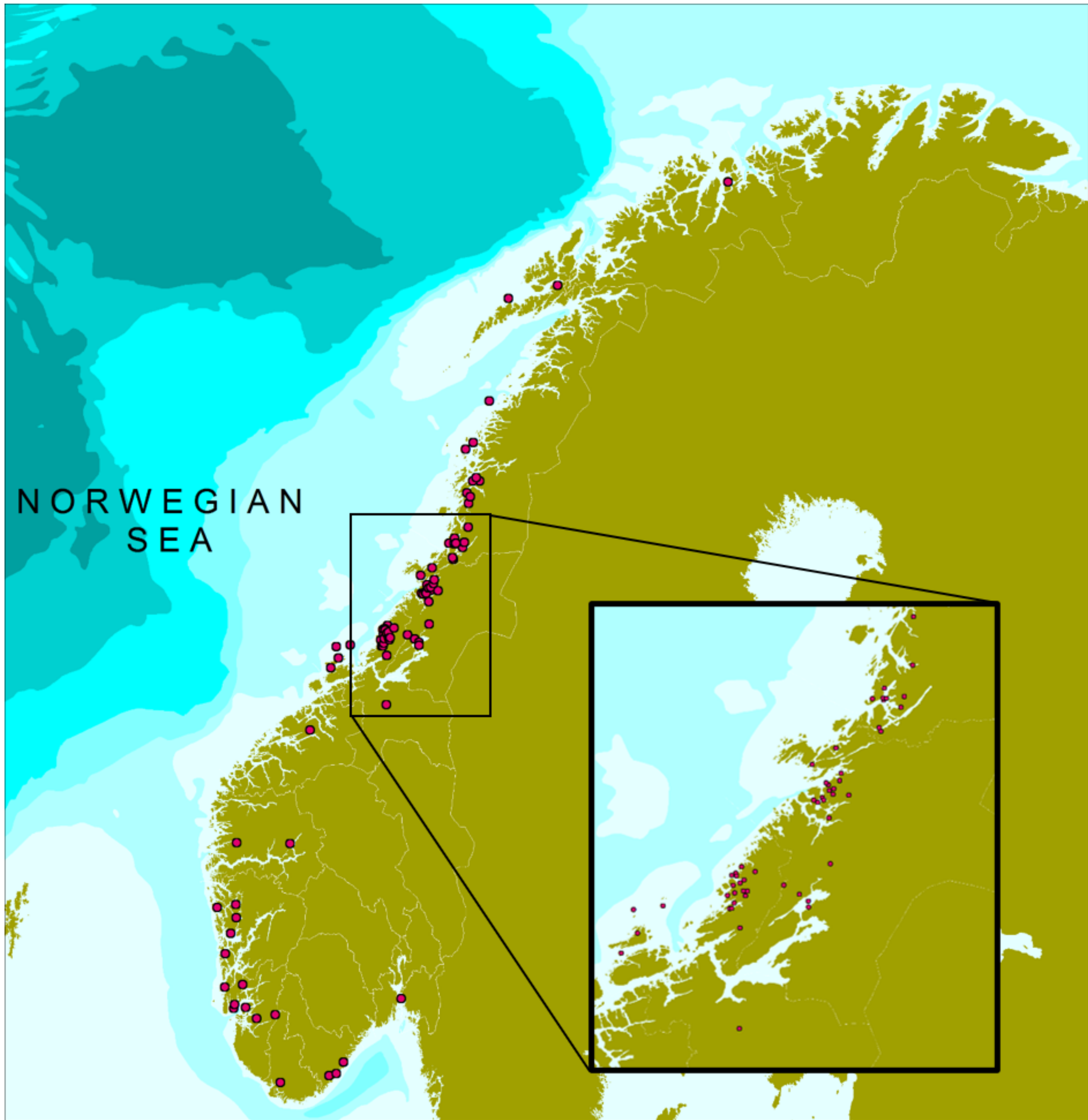

Figure 2.2: Map showing sampling sites of the bivalve mollusc samples. Samples from Trøndelag are enlarged in the bottom right corner.

2.2 Detection, enumeration, and verification

2.2.1 Preparation of sample dilutions for the Most Probable Number (MPN) method

For the detection and enumeration of enterococci in the bivalve samples, the Most Probable Number (MPN) method APHA 2001 for enterococci and faecal streptococci in foods (Hartman et al., 2001; Zimbro & Power, 2003) was used (Fig. 2.3). From each sample, the ten largest individuals were selected. Dead individuals were excluded. Individuals were scrubbed free from dirt and barnacles in cold water. After rinsing, bivalves were put on paper towels on a tray. With a rounded knife, each bivalve was cut open, and the flesh and intravalvular liquid collected into separate stomacher bags. Each bag contained more than 50 grams of material and was homogenised for 2.5 minutes at 4 strokes per second in a stomacher machine (Interscience BagMixer® 400). From the homogenate, 50 grams was poured into a new stomacher bag with filter and added 100 grams of peptone water by a dispenser machine (Dilumat™ Blueline 3125). The sample was homogenized for 2.5 minutes at 185 rpm in a stomacher machine (Stomacher® 400 CIRCULATOR, Seward) prior to adding additional 350 grams of peptone water, making a 1:10 dilution. From the 1:10 dilution, a 1:100 dilution was made by transferring 10 mL into a new stomacher bag, adding 90 mL of peptone water. These two dilutions were used in the MPN method for the enumeration and quantification of *Enterococcus* in the bivalve samples.

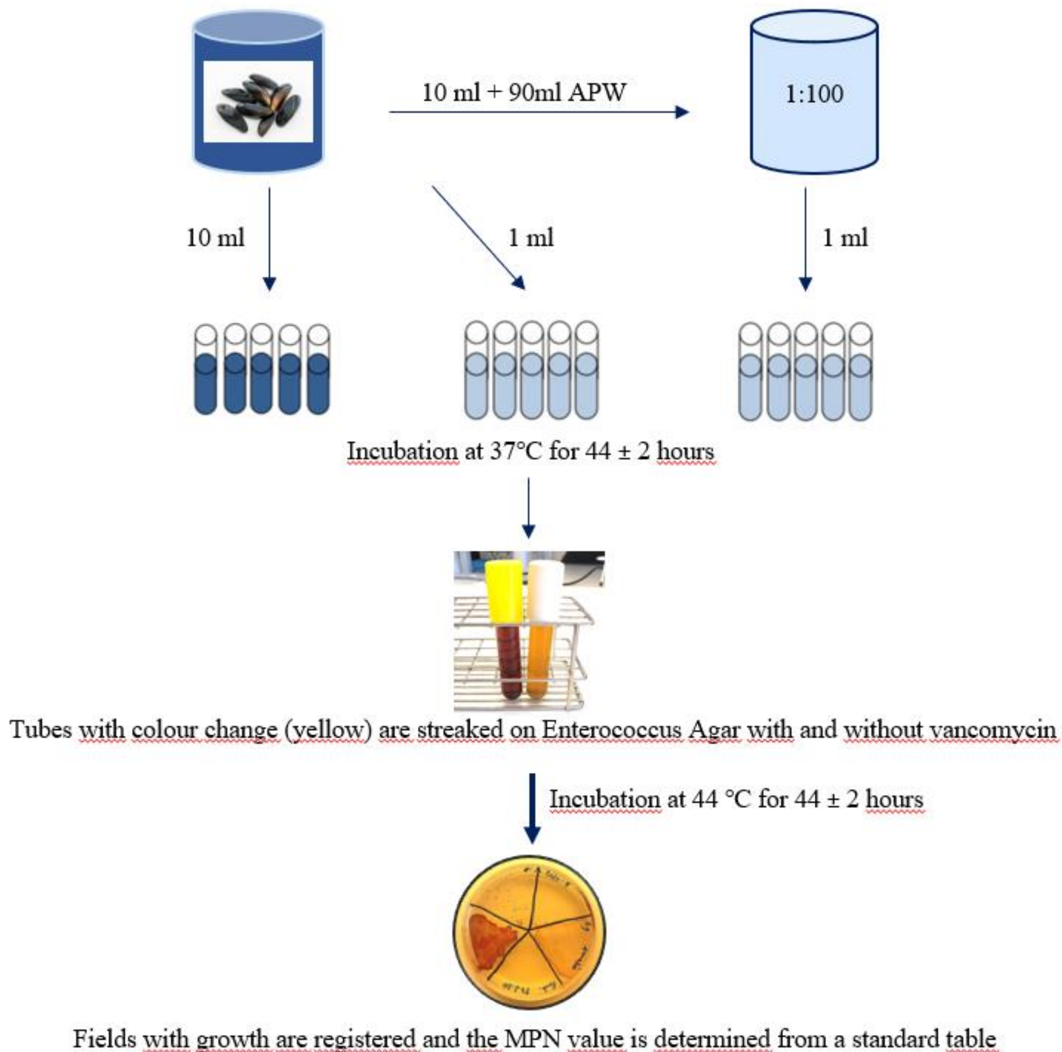

Figure 2.3: The MPN method illustrated. From the original sample, threefold dilutions were made and incubated.

2.2.2 MPN method for detection and enumeration

From the two diluted bivalve samples, three dilutions (1:1, 1:10, 1:100) consisting of five tubes each were prepared for each sample comprising 1 gram, 0.1 grams and 0.01 grams sample material. The 1:1 dilution consisted of 10 mL from the initial 1:10 dilution in 10 mL double strength KF Streptococcus Broth (BD Difco™), the 1:10 dilution consisted of 1 mL from the initial 1:10 dilution in 10 mL single strength Streptococcus Broth, and the 1:100 dilution consisted of 1mL from the initial 1:100 dilution in 10 mL single strength Streptococcus Broth. Tubes were incubated for 48 ± 2 hours at 37 ± 1°C.

Table 2.1 The table shows how the different dilutions were made for the MPN method.

MPN dilution	Sample/dilution	Aliquot from 1:10 dilution bag	Aliquot from 1:100 dilution bag	mL KF Streptococcus Broth
1:1	1 gram	10 mL	-	10 mL double strength
1:10	0.1 grams	1 mL	-	10 mL single strength
1:100	0.01 grams	-	1 mL	10 mL single strength

2.2.3 Verification of enterococcal isolates on selective agar

After incubation in KF Streptococcus Broth, tubes containing enterococci would show a change in colour from dark red to ochre yellow (Figure 2.4). To confirm the actual presence of enterococci in the sample, 10 µl of the tube content was streaked on enterococci-selective agar plates (*Enterococcus* Agar, BD Difco™) and incubated in water bath for 48 ± 2 hours at 44 ± 1°C (NMKL, 2011). The presence of enterococci in the sample appeared as pink and purple colonies with diameters varying from 0.5 to 2 millimetres. The presence of enterococci in each tube for each of the three dilutions was registered and the MPN values for each sample could be obtained from the standardized MPN table. The *E. faecalis* strain CCUG 9997 was used as a positive control for the Streptococcus Broth, *Enterococcus* agar and Luria Bertani (LB) agar (MP Biomedicals).

Figure 2.4 After incubation, tubes containing enterococci would change colour from dark red (left) to ochre yellow (right).

2.3 Characterisation of isolates

2.3.1 Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry

For the species characterisation of the bacterial isolates, each frozen isolate was streaked on LB agar plates and incubated in water bath for 48 ± 2 hours at 44 ± 1°C to obtain fresh colonies. Using an aseptic toothpick, a sufficient amount of bacterial material was transferred to one spot on the target plate. The target plate was set to dry at room temperature inside a sterile bench for 2-3 minutes. On each spot of the plate, 1 µl of the matrix α-cyano-4-Hydroxycinnamic Acid (HCCA) (Bruker, Germany) was added to specifically absorb the laser beam. After the matrix had dried, the target plate was placed in the MALDI-TOF (Microflex® LRF) instrument (Bruker, Germany) and analysed. Results were registered and interpreted via the FlexControl (Bruker, Germany) program.

2.4 Antimicrobial susceptibility testing

2.4.1 Screening for vancomycin resistance using agar selective methods

While doing the MPN method, the bivalve samples were also tested for the presence of vancomycin resistant enterococci. From the tubes that showed a change in colour from dark red to ochre yellow after incubation, 10 µl were streaked on *Enterococcus* agar plates containing vancomycin (6 mg/L). Plates were incubated in water bath for 48 ± 2 hours at 44 ± 1°C. The presence of vancomycin resistant enterococci would be confirmed by the formation of pink or purple bacterial colonies on the agar after incubation.

2.4.2 Antimicrobial susceptibility testing using the microdilution method

The antimicrobial susceptibility testing followed the International Standard ISO 20776-1 by the European Committee on Antimicrobial Susceptibility Testing (International Organization for Standardization, 2019). The Sensititre™ Gram Positive MIC 96-well Microplates "EUENCF" (Thermo Fischer™) were used to test for antimicrobial resistance in the enterococcal isolates.

Before testing, each frozen isolate was streaked on LB agar and incubated in water bath for 48 ± 2 hours at 44 ± 1°C to obtain fresh colonies. The following procedure was done the same way for each enterococcal isolate: a bacterial suspension with a McFarland standard turbidity of 0.5 was made by picking 1-3 colonies (depending on the colony size) into a test tube with an aliquot of 2 ml sterile saline water (0.90 % NaCl). The bacterial suspension was vortexed, and 30 µl was transferred into a tube containing 11 mL Mueller Hinton broth (Thermo Fischer ™). The desired inoculum concentration was 1 x 10^5 cfu/ml. The Mueller Hinton broth tube was vortexed before putting on a dosing head (Thermo Fischer™) and placed in the dosing machine (Sensititre AIM ™ Automated Inoculation Delivery System, Thermo Fischer ™). The dosing volume was set to 50 µl. After dosing, each Sensititre plate was sealed with a plate sealing film and incubated aerobically for 24 ± 2 hours at 37 ± 1°C. The time from when the colony was suspended in the saline water until the microtiter plate was incubated, took less than 15 minutes.

Each isolate was tested for 15 different antimicrobials, covering 10 different antimicrobial classes (Table 2.1). After incubation, Sensititre plates were loaded into the Sensititre™ Vizion™ Digital MIC Viewing System (Thermo Fischer ™) and MIC values determined manually in the Sensititre™ SWIN™ Software System (Thermo Fischer ™) on a computer. Results were based on EUCAST breakpoints for *Enterococcus* spp. (EUCAST, 2021).

Table 2.1 List of antimicrobials in the EUENCF microtiter plate and their MIC breakpoints.

Antimicrobial class	Substance	Concentrations (min.-max., mM)	MIC Breakpoints	
			S ≤	R >
Penicillins	Ampicillin	0.25 - 32	4	8
	Amoxicillin	0.25 - 32	4	8
	Amoxicillin / clavulanic acid	0.25/2 - 32/2	4	8
Glycopeptides	Vancomycin	0.12 - 16	4	4
	Teicoplanin	0.5 - 8	2	2
Trimethoprim	Trimethoprim	0.015 - 2		
Oxazolidinone	Linezolid	0.5 - 8	4	4
Nitrofuran	Nitrofurantoin	32 - 64	64 (*E. faecalis*)	64 (*E. faecalis*)
Aminoglycosides	Streptomycin	512 - 1024		
	Gentamicin	32 - 256		
Carbapenem	Imipenem	0.5 - 16	0.001	4
Streptogramin	Q/D	0.25 - 8	1 (*E. faecium*)	4 (*E. faecium*)
Glycylcycline	Tigecycline	0.06 - 2	0.25	0.25
Fluoroquinolones	Ciprofloxacin	0.5 - 16	4	4
	Levofloxacin	0.5 - 16	4	4
	Norfloxacin	4 - 16	NA	NA

2.5 Heavy metal susceptibility testing

For the heavy metal susceptibility testing, different methods were evaluated before the final method was established.

2.5.1 Microdilution method

Stock solutions of each heavy metal were made by dissolving copper sulphate pentahydrate ($CuSO_4 \cdot 5H_2O$), zinc sulphate heptahydrate ($ZnSO_4 \cdot 7H_2O$) and cadmium chloride ($CdCl_2$) in Mueller Hinton Broth. Copper and sink stock solutions had molar concentrations of 80 millimolar, and the cadmium chloride stock solution had a molar concentration of 40 millimolar. From each stock solution, eleven dilutions were made and sterile filtered into sterile glass tubes with black caps (re-used Mueller Hinton Broth tubes from Thermo Fischer™) and

stored at 4°C. The black caps were replaced by dosing heads (Thermo Fischer ™) when preparing the dilutions in the microtiter plates in the dosing machine by Thermo Fischer™. In a 96-well microtiter plate, columns 1 to 11 were filled with each of the eleven dilutions, from the lowest molar concentration to the highest (Fig. 2.5). The 12th column served as a positive control for the bacterial sample. In each row (A-I), suspensions of enterococci in Mueller Hinton Broth were added. In the last row (J), only Mueller Hinton Broth was added, and this row served as a sterile control for the heavy metal dilutions and the Mueller Hinton Broth. The microtiter plates were sealed with a plate sealing film and incubated aerobically for 24 ± 2 hours at 37 ± 1°C. After incubation, the plates were assessed to determine a MIC value with the naked eye or with a magnifier. The microtiter plates were also scanned for absorbance in a Victor™ X5 2030 Multilabel Reader (Perkin Elmer, USA) applying filters at 340, 380, 405, 450, 492, 531, 540, 570, 620 and 690 nm to find a suitable wavelength that would absorb cell growth only, and not metal pigmentation.

Figure 2.5: 96-well microtiter plate filled with serial dilutions of copper sulphate pentahydrate, starting with the highest concentration in the left column.

2.5.2 Agar dilution method

For the agar dilution method, a dilution series was made for each heavy metal. One stock solution was prepared, consisting of the heavy metal dissolved in sterile water

For copper sulphate pentahydrate and zinc sulphate heptahydrate, agar plates with molar concentrations of 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32 and 40 mM were made (Fig. 2.6). For cadmium chloride, the agar plates had molar concentrations of 0.03, 0.06, 0.125, 0.25, 0.5, 1, 2, 4, 8 and 16 mM (Fig. 2.7). Ten flasks (A-J) with adjusted volumes of sterile Mueller Hinton agar were prepared and at lukewarm temperatures, the appropriate volumes from a pre-heated

(37 °C) heavy metal stock solution were added to the agar. The pH was adjusted to ~7 using 4N Sodium Hydroxide (NaOH) and 6N Hydrogen Chloride (HCl). From each heavy metal agar, a small sample from four of the dilutions was sent to the inorganic lab at the IMR for measuring the metal concentrations in the agar.

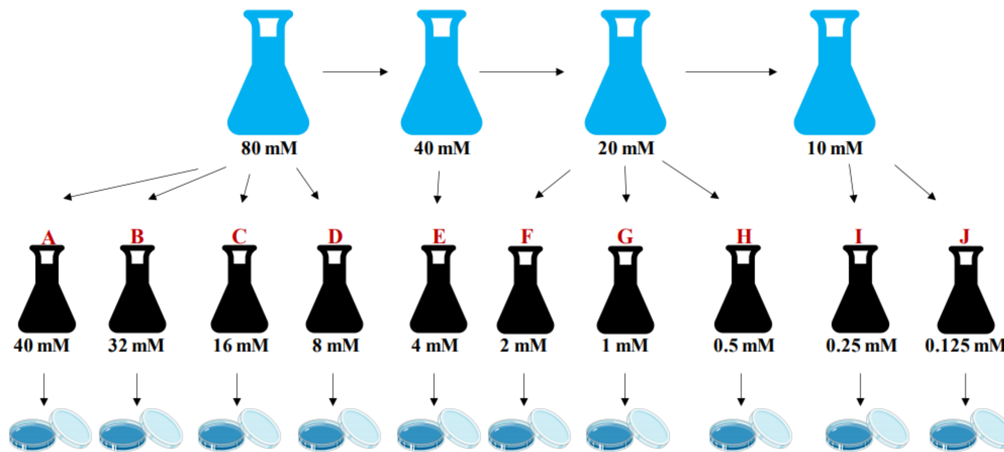

Figure 2.6 Overview of how copper and zinc agar dilutions (A-J) were made from copper and zinc stock solutions (blue flasks) together with Mueller Hinton agar.

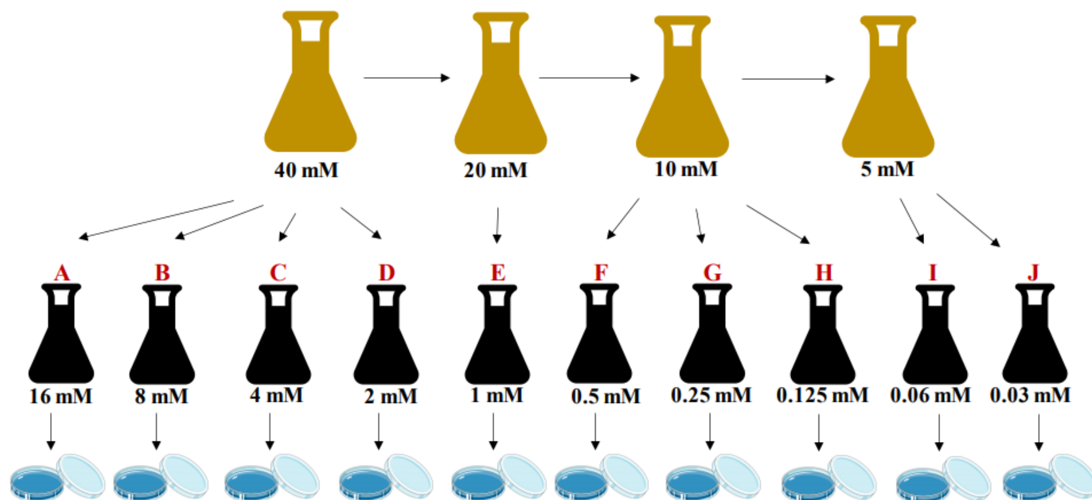

Figure 2.7 Overview of how cadmium agar dilutions (A-J) were made from cadmium stock solutions (blue flasks) together with Mueller Hinton agar.

The largest challenge was preventing the heavy metal from precipitating. The heavy metal stock solutions could not be autoclaved as the high temperatures would trigger precipitation, thus sterility controls were done by incubating plates containing only heavy metal agar for 48 ± 2 hours at 44 ± 1°C in water bath to see if there was any growth.

2.5.3 Minimum Inhibitory Concentration (MIC) for heavy metals and breakpoints

The heavy metal agar plates were used to determine MIC values for each metal. All 480 enterococcal isolates were tested for susceptibility to copper, zinc, and cadmium. Isolates were grown on LB agar for 48 ± 2 hours at 44 ± 1°C in water bath prior to preparation of inoculum where cell material was collected using a sterile cotton swab and mixed with 5 mL physiological saline (0.9% NaCl) in a sample tube to give a McFarland standard turbidity of 1.0. The cotton swab was placed on its respective spot (1-51) on each plate, dipping the swab into the bacterial inoculum before placing every new spot (Fig. 2.8). *E. faecalis* CCUG 9997 was included on each plate as control. This procedure was repeated for all 480 isolates. The heavy metal agar plates were incubated for 48 ± 2 hours at 37 ± 1°C. Since there are no standardized breakpoints given for heavy metals as of today (Norwegian Food Authority, 2014), breakpoints for copper, zinc and cadmium were determined based on this and previous studies.

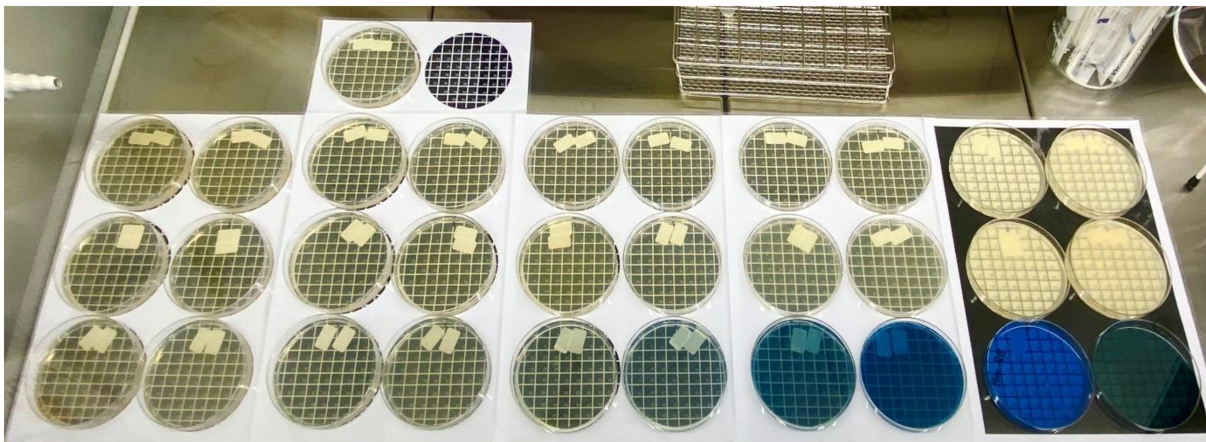

Figure 2.8: Agar plates were aligned on laminated sheets indicating spot numbers 1-52. Top row from left to right: cadmium plates from 0.03 to 16 mM; middle row: zinc plates from 0.125 to 40 mM and bottom row: copper plates from 0.125 to 40 mM. Single plate on top: MHA without metal.

After incubation, MIC values were registered and epidemiological cut-off values (ECOFFs) for each heavy metal were determined using the ECOFFinder estimation sheet provided by EUCAST and CLSI (Turnidge et al., 2006). Breakpoints with 95.0 % confidence intervals for copper, zinc and cadmium were estimated to be 32 mM, 64 mM, and 1 mM, respectively. For the estimation of the breakpoint values, the results from certain species of *Enterococcus* were omitted in the calculation process due to low numbers of isolates for these species (Table 2.2).

Table 2.2: An overview of the estimated epidemiological cut-off values (ECOFFs) for copper, zinc, and cadmium with a 95.0% confidence interval (CI) and which species' MIC values were used for calculating the ECOFFs. The number of isolates used in total to calculate the ECOFFs are indicated in column four. Due to a low number of isolates from certain species, these were not included in calculating the breakpoints, as seen in column five.

Heavy metal	ECOFF (mg/L) 95.0% CI	Based on results from	Number of isolates	Not included in calculations
Copper	32	*E. faecium* *E. hirae* *E. faecalis* *E. casseliflavus*	438	*E. avium* *E. durans* *E. gallinarum* *E. mundtii* *E. thailandicus* *E. villorum*
Zinc	64	*E. faecium* *E. hirae* *E. faecalis* *E. casseliflavus*	438	*E. avium* *E. durans* *E. gallinarum* *E. mundtii* *E. thailandicus* *E. villorum*
Cadmium	1	*E. faecium* *E. hirae* *E. faecalis* *E. casseliflavus*	438	*E. avium* *E. gallinarum* *E. mundtii* *E. thailandicus* *E. villorum*

2.6 Molecular methods

2.6.1 Extraction of DNA using DNeasy® Blood & Tissue Kit

Isolates were grown on LB agar for 48 ± 2 hours at 44 ± 1°C in water bath. The DNA was extracted according to the protocol in the DNeasy® Blood & Tissue kit, beginning with the pre-treatment for Gram-Positive Bacteria (Qiagen, Germany). With a sterile 10 µl loop, round and separately growing colonies were picked and transferred to a 1.5 mL Eppendorf tube containing 500 µl of Phosphate Buffered Saline (PBS) (Sigma-Aldrich, USA). The tubes were centrifuged for 10 minutes at 5000 x g. The supernatant was removed, and 500 µl PBS were added again before centrifuging a second time for 10 minutes at 5000 x g. The supernatant was removed, and 180 µl enzymatic lysozyme buffer (20 mg lysozyme/mL lysis buffer) was added. Samples were vortexed and placed in a heating block for 30 minutes at 37°C with the shaking set to 1000 rpm. Samples were regularly vortexed during incubation. After incubation, 20 µl RNase, 200 µl Buffer AL and 25 µl Proteinase K was added and the tubes were vortexed. Tubes were incubated again for 30 minutes at 56°C with the shaking set to 1000 rpm with regularly vortexing during incubation. After incubation, 200 µl 100% ethanol was added and tubes were vortexed.

After the pre-treatment, the content (~630 µl) of each tube was transferred to a DNeasy Mini spin column which was placed in a DNeasy collection tube. The samples were centrifuged for 1 minute at 6000 x g. The DNeasy spin column was placed in a new 2 mL DNeasy collection tube and 500 µl Buffer AW1 was added. The samples were centrifuged for 1 minute at 6000 x g. The DNeasy spin column was placed in a new 2 mL DNeasy collection tube and 500 µl Buffer AW2 was added. The samples were centrifuged for 2 minutes at 20 000 x g, followed by a short break and another centrifugation for 1 minute at 17 000 x g. The DNeasy Mini spin column was carefully placed into a sterile 1.5 mL Eppendorf tube. 50 µl Buffer EB was added at the centre of the membrane. The samples were incubated at room temperature in 1 minute before centrifuging them for 1 minute at 6000 x g. A second eluate was made by placing the DNeasy Mini spin column into a new 1.5 mL Eppendorf tube, adding 30 µl Buffer EB and centrifuging for 1 minute at 8000 rpm. The DNA extracts were stored at -20°C.

2.6.2 Nanodrop and Qubit

Nucleic acids in DNA and RNA usually show an absorbance maximum at 260 nm and 280 nm, respectively (Matlock, 2015). To measure the purity in a DNA or RNA sample, the ratio of the absorbance maximum to the absorbance at 280 nm is calculated. The optimal 260/280 ratio is ~1.8 DNA extracts and ~2.0 for RNA extracts (Matlock, 2015). Contamination of the sample

with residual phenol or other reagents from the extraction protocol will usually give a too low 260/280 ratio. The 260/230 ratio is measured to check for contamination in the sample and should lie within 2.0 and 2.2. The purity ratios 260/280 and 260/230 were measured from the DNA elutes using Nanodrop (Nanodrop ND-1000 Spectrophotometer, Nanodrop Technologies, USA). Qubit (Qubit 2.0 Fluorometer, Invitrogen) was used to measure the concentration of DNA in the sample.

2.7 Whole Genome Sequencing (WGS)

For the whole genome sequencing of the bacterial isolates, the Illumina Sequencing Technology was used.

2.7.1 Sequencing for studying antimicrobial resistance genes (Tromsø)

Four *E. faecium* isolates that expressed resistance towards ampicillin, gentamicin and Q/D were whole genome sequenced at Genomics Support Center Tromsø at UiT using Illumina Sequencing Technology. Libraries were prepared by the Nextera XT DNA library preparation kit (Illumina, San Diego, USA) and sequenced using Illumina NextSeq500 and the Mid Output 300 cycles cell. The obtained raw sequence data was processed at K-res and the IMR.

2.7.2 Bioinformatic analyses at K-res

Adapter removal and quality trimming of the raw reads were performed by trimmomatic v0.39 (Bolger et al., 2014). Later, genome assembly was done using SPAdes v3.13.0 (Bankevich et al., 2012) and the quality of assembled genomes was assessed using QUAST v5.0.2 (Gurevich et al., 2013). Antimicrobial resistance genes were identified *in silico* from the assemblies using NCBI bacterial AMR reference gene database (PRJNA313047) (Feldgarden et al., 2019) in ABRicate tool (Seemann, 2020) v0.8.7. To explore the phylogenetic relationship between the marine *E. faecium* and publicly available genome sequences on NCBI, a global phylogenetic tree was generated based on the core genome. Closed genomes of *E. faecium* (n=154) from NCBI were retrieved and phylogenetic trees were constructed using Parsnp v1.2 (Treangen et al., 2014). Multilocus Sequence Typing (MLST) was performed using MLST tool v2.11 (Jolley & Maiden, 2010). For high-resolution typing, Minimum Spanning Tree was generated based on the 1423 core genes of *E. faecium* scheme of SeqSphere+ software v6.0.2 (Ridom GmbH, 2020). We used the default ≤ 20 allelic differences as a threshold for cluster calculation and clonal relatedness of *E. faecium* (De Been et al., 2015).

2.7.3 Bioinformatic analyses at IMR

The FASTA/assembly files of the four *E. faecium* isolates were run through the BacMet database v2.0 (Pal et al., 2014) using a 90% similarity match to the "Predicted Database" for metal resistance genes.

2.8 Culture collection

2.8.1 Freezing isolates in pure culture for storage

A colony was picked from each enterococci-positive tube in the MPN method, streaked on *Enterococcus* agar (BD Difco™) and re-streaked 2-3 times on Luria Bertani agar (MP Biomedicals) to ensure a pure culture of the bacterial cells. The agar plates were incubated in water bath for 48 ± 2 hours at 44 ± 1°C, before picking fresh colonies for freezing down with glycerol in Eppendorf tubes at -80°C.

2.9 Statistics and preparations of graphs

For making figures and maps, Microsoft Excel, PowerPoint and MapInfo Professional v10.0 was used, tables were made in Microsoft Excel. Confidence interval (95%) was calculated by binominal test in R Studio. Seasonality tests and box plots were made in GraphPad Prism v.8.2.1 performing Kruskal-Wallis test with non-parametric mean rank differences and Dunn's multiple comparisons test.

3. Results

3.1 Sampling and samples

The 473 bivalve mollusc samples were collected from 89 different geographical locations in seven different counties (Table 3.1), and the sample species were blue mussel (*Mytilus edulis*), European flat oyster (*Ostrea edulis*), great scallop (*Pecten maximus*), horse mussel (*Modiolus modiolus*), ocean quahog (*Arctica islandica*), carpet shell (*Polititapes rhomboides*), sea urchin (*Strongylocentrotus droebachiensis*), cockle (*Cardiidae*) and pacific oyster (*Magallana gigas*).

Table 3.1: The 473 bivalve samples consisted of eight different sample species and were sampled from seven counties as shown in the table. The percentage of each sample species collected in each county is indicated in parentheses.

Species	Agd. (%)	Nord. (%)	Rog. (%)	T&F (%)	Tr. (%)	V&T (%)	Vestl. (%)	Total
Blue mussel	16 (89)	82 (98)	14 (38)	12 (100)	227 (96)	-	39 (46)	390
European flat oyster	1 (5.5)	-	8 (22)	-	-	-	35 (42%)	44
Great scallop	-	2 (2)	10 (27)	-	9 (4)	-	5 (6)	26
Horse mussel	-	-	3 (8)	-	1 (0)	-	-	4
Ocean quahog	-	-	-	-	-	-	3 (4)	3
Sea urchin	-	-	2 (5)	-	-	-	-	2
Carpet shell	-	-	-	-	-	-	2 (2)	2
Cockle	1 (5.5)	-	-	-	-	-	-	1
Pacific oyster	-	-	-	-	-	1 (100)	-	1
Total (n and (%))	18 (4)	84 (18)	37 (8)	12 (2)	237 (50)	1 (0)	84 (18)	**473**

Agd.=Agder, Nord.=Nordland, Rog.=Rogaland, T&F=Troms & Finnmark, Tr.=Trøndelag, V&T=Vestfold & Telemark, Vestl.=Vestland.

3.2 Detection of presumptive enterococci in bivalve molluscs

3.2.1 Prevalence and concentrations of enterococci

Among the 473 sample, 286 (60 ± 4.6%, 95% CI) were positive for the presence of presumptive enterococci. These samples originated from 71 different locations spread across 35 municipalities in the six counties Agder, Nordland, Rogaland, Troms & Finnmark, Trøndelag and Vestland. The single sample from Vestfold & Telemark was negative. On average one isolate was isolated from different enrichments belonging to the sample. The isolate collection comprised 480 presumptive enterococcal isolates. Among the 286 samples that carried presumptive enterococci, the enterococcal concentrations ranged from <18 to 3500 MPN/100g,

with a median concentration of 18 MPN/100g. The highest concentration at 3500 MPN/100g was detected in a sample of blue mussels, sampled in June in 2020. 87 % of the samples had concentrations below 100 MPN/100g.

The composition of *Enterococcus* species differed in the bivalve samples. Among the 286 samples, 253 (88%) contained isolates belonging to one *Enterococcus* species, 29 (10%) to two species, three (1%) contained three species (Table 3.2) and one (0.3%) sample (2020-1129) contained four *Enterococcus* species: *E. faecium*, *E. faecalis*, *E. hirae* and *E. mundtii* (Table 3.2).

Table 3.2: The four samples that contained the highest number of different *Enterococcus* species.

Sample No.	No. *Enterococcus* species (n)	From bivalve species	*Enterococcus* species
2020-1129	4	Blue mussel	*E. faecium, E.faecalis, E. hirae, E. mundtii*
2020-331	3	Blue mussel	*E. faecium, E. faecalis, E. hirae*
2020-1126	3	Blue mussel	*E. faecium, E. faecalis, E. thailandicus*
2020-1123	3	Blue mussel	*E. faecium, E. durans, E. hirae*

In the 29 samples containing two different species, *E. faecium* and *E. hirae* were most frequently observed together (Table 3.3).

Table 3.3: The nine different combinations of two enterococcal species that was seen in 29 samples.

Combination of *Enterococcus* pairs	No. of samples
E. faecium and *E. hirae*	15
E. faecium and *E. faecalis*	5
E. faecium and *E. durans*	2
E. faecalis and *E. hirae*	2
E. faecalis and *E. durans*	1
E. faecalis and *E. mundtii*	1
E. hirae and *E. casseliflavus*	1
E. hirae and *E. mundtii*	1
E. thailandicus and *E. casseliflavus*	1

3.2.2 Faecal pollution in bivalve samples

The concentrations of enterococci were correlated with the concentrations of *E. coli* in the same samples, and no correlation was found between the two MPN values (Fig. 3.1). This was based on comparison of MPN values of 201 of the 473 (42%) bivalve mollusc samples. The correlation value was -0.10141. There was no correlation between the *E. coli* and enterococci MPN values for the samples which the four sequenced *E. faecium* came from: isolates 2020-755/3, 2020-756/3, 2020-523/1 and 2020-324/5 had enterococcal MPN values of 68, 68, 490 and 20, respectively, and the *E. coli* MPN value for all these samples was <18, the lowest achievable MPN value. A significant difference between summer and spring (P value = 0.0309) and summer and autumn (P value = 0.0335) (Fig. 3.2) was found.

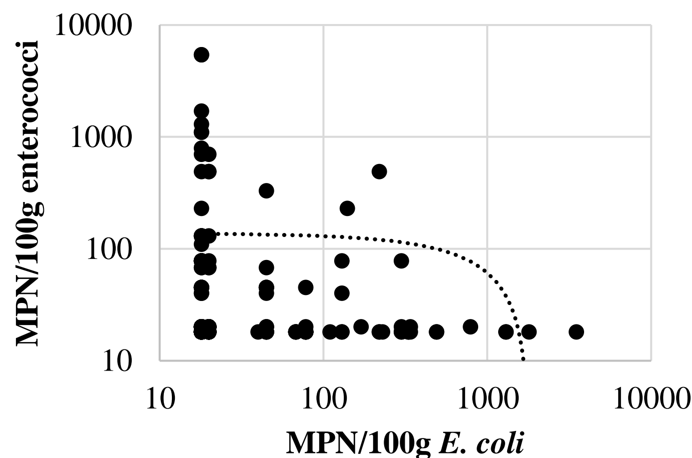

Figure 3.1: A correlation figure between the MPN values of 201 samples that had MPN data available for both enterococci and *E. coli*. There was no positive correlation between the two datasets (y=-0.0768x + 137.58).

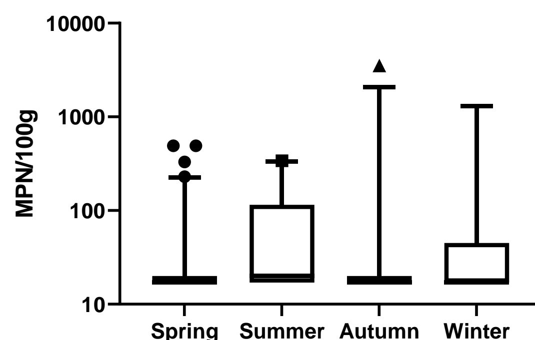

Figure 3.2: Concentrations of enterococci (MPN/100g) divided into seasons. The logarithmic Y-axis indicates the concentration of enterococci as MPN/100g. X-axis indicates the seasons Spring (March-May), summer (June-August), autumn (September-November) and winter (December-February). Boxes indicate the 25-75 % percentile, whiskers indicate the 5-95 % percentile. Symbols (● ■ ▲) indicate values above the 5-95 % percentile.

3.3 Identification of ten enterococcal species among isolates

Among the 480 presumptive enterococci isolates, the following species were identified: *E. faecium*, *E. hirae*, *E. faecalis*, *E. durans, E. casseliflavus*, *E. avium, E. thailandicus*, *E. gallinarum*, *E. mundtii, E. villorum* and isolates *Enterococcus* spp. with unidentified species (Table 3.2).

Table 3.2: The total of 480 isolates were isolated from six different bivalve species. MALDI-TOF MS analyses found that the isolates belonged to ten different *Enterococcus* species. The number of isolates of each *Enterococcus* species found in the different bivalve species is indicated in percent.

Species	Bm	Efo	Gs	Hm	Bcs	Co	Total
***E. faecium* (%)**	207 (84)	23 (9)	15 (6)	1 (0.4)	1 (0.4)	-	247
***E. hirae* (%)**	102 (89)	5 (4)	6 (5)	1 (1)	-	1 (1)	115
***E. faecalis* (%)**	53 (80)	11 (17)	2 (3)	-	-	-	66
***E. durans* (%)**	25 (100)	-	-	-	-	-	25
***E. casseliflavus* (%)**	7 (100)	-	-	-	-	-	7
***E. avium* (%)**	3 (75)	-	-	1 (25)	-	-	4
***E. thailandicus* (%)**	4 (100)	-	-	-	-	-	4
***E. gallinarum* (%)**	3 (100)	-	-	-	-	-	3
***E. mundtii* (%)**	3 (100%)	-	-	-	-	-	3
***E. villorum* (%)**	1 (100%)	-	-	-	-	-	1
***Enterococcus* spp. (%)**	5 (100%)	-	-	-	-	-	5
Total	413	39	23	3	1	1	480

Bm=blue mussel, Efo=European flat oyster, Gs=Great scallop, Bcs=Banded carpet shell, Hm=horse mussel, Co=Cockle.

3.4 Detection of antimicrobial and heavy metal resistant isolates

3.4.1 Resistance to twelve antimicrobials was observed

No growth was observed when enrichment from the MPN method was spread on the surface of *Enterococcus*-selective agar plates containing vancomycin (6 mg/L). Antimicrobial susceptibility testing by the microdilution method showed that 88 (18%) (Fig. 3.4) of the 480 isolates expressed resistance to one or more of the antimicrobial agents (Table 3.3), with the highest prevalence among *E. faecium* (76%).

Table 3.3. The prevalence of antimicrobial resistance in all tested enterococcal species.

Species	No. of isolates (n)	No. of isolates resistant to one or more AMs (% of AMR isolates)
E. faecium	247	67 (76)
E. faecalis	66	7 (8)
E. casseliflavus	7	3 (3.4)
E. avium	4	3 (3.4)
E. gallinarum	3	3 (3.4)
E. durans	25	1 (1.1)
E. hirae	115	1 (1.1)
E. thailandicus	4	1 (1.1)
E. mundtii	3	0
E. villorum	1	0
Enterococcus spp.	5	2 (2.3)
Total	480	88 (18% of all isolates)

AM=Antimicrobial, AMR=Antimicrobial Resistant

Among the 88 resistant isolates, resistance towards fluoroquinolones (69%), streptogramins (16%), trimethoprim (16%), aminoglycosides (19%), imipenem (17%), glycopeptides (7%), penicillins (6%) and glycylcyclines (2%) was observed (Table 3.4). None of the isolates showed resistance to the oxazolidinone linezolid or nitrofurantoin. Of the total collection of enterococcal isolates, 392 (82%) were sensitive and expressed no resistance to the antimicrobials mentioned above.

Table 3.4 Antimicrobial resistance in enterococcal isolates recovered from bivalve samples.

	E. faecium (247)	*E. hirae (115)*	*E. faecalis (66)*	**Others[1] (27)**	**Total**
Amoxicillin	3	0	0	2	5
AMOCLA	3	0	0	2	5
Ampicillin	3	0	0	2	5
Ciprofloxacin	36	0	1	3	40
Gentamicin	2	0	2	1	5
Imipenem	11	1	1	2	15
Levofloxacin	22	0	0	3	25
Linezolid	0	0	0	0	0
Nitrofurantoin	0	0	0	0	0
Norfloxacin	43	0	1	5	49
Q/D	4	0	0	4	8
Streptomycin	9	1	2	3	15
Teicoplanin	0	0	0	1	1
Tigecyclin	1	0	0	1	2
Trimethoprim	19	0	0	3	22
Vancomycin	0	0	0	6	6

[1]Others: E. avium, E. durans, E. casseliflavus, E. gallinarum, E. mundtii, E. thailandicus, E. villorum and Enterococcus spp. AMOCLA=Amoxicillin/Clavulanic acid, Q/D=Quinupristin/dalfopristin.

3.4.2 Antimicrobial resistant enterococci were found in all counties

From the 88 resistant isolates (Fig. 3.3), 55 (40%) derived from samples collected in Trøndelag, 34 (25%) from Nordland, 22 (16%) from Vestland, 15 (11%) from Agder, 8 (6%) from Rogaland and 2 (2%) from Troms & Finnmark (Table 3.5).

Table 3.5. An overview of how many samples were sampled, the number of samples containing enterococci/antimicrobial resistant enterococci, the number of enterococcal/antimicrobial resistant enterococcal isolates and the proportion of enterococci/antimicrobial resistant enterococci found per sample per county. Numbers for antimicrobial resistance are highlighted in orange.

County	Samples (n)	No. samples with ent. (n)	Samples with ent. (%)	No. ent. isolates (n)	Proportion of ent. per sample	No. samples with AMR ent. (n) (%)	No. AMR isolates (n)	Proportion of AMR ent. per sample
Agd.	18	14	78	26	1.4	4 (22)	10	0.56
Nordl.	84	47	56	101	1.2	15 (18)	20	0.24
Rog.	37	24	65	30	0.8	4 (11)	4	0.11
T&F	12	8	67	12	1.0	2 (17)	2	0.17
Tr.	237	146	62	242	1.0	37 (16)	43	0.18
V&T	1	-	-	0	0	0	0	0
Vestl.	84	47	56	69	0.8	7 (8)	9	0.11
Tot.	473	286	60	480	6.2	69 (15)	88	0.19

AMR=Antimicrobial resistant, ent.=enterococci, Agd=Agder, Nordl.=Nordland, Rog.=Rogaland, T&F=Troms & Finnmark, Tr.=Trøndelag, V&T=Vestfold og Telemark, Vestl.=Vestland, Tot.=Total.

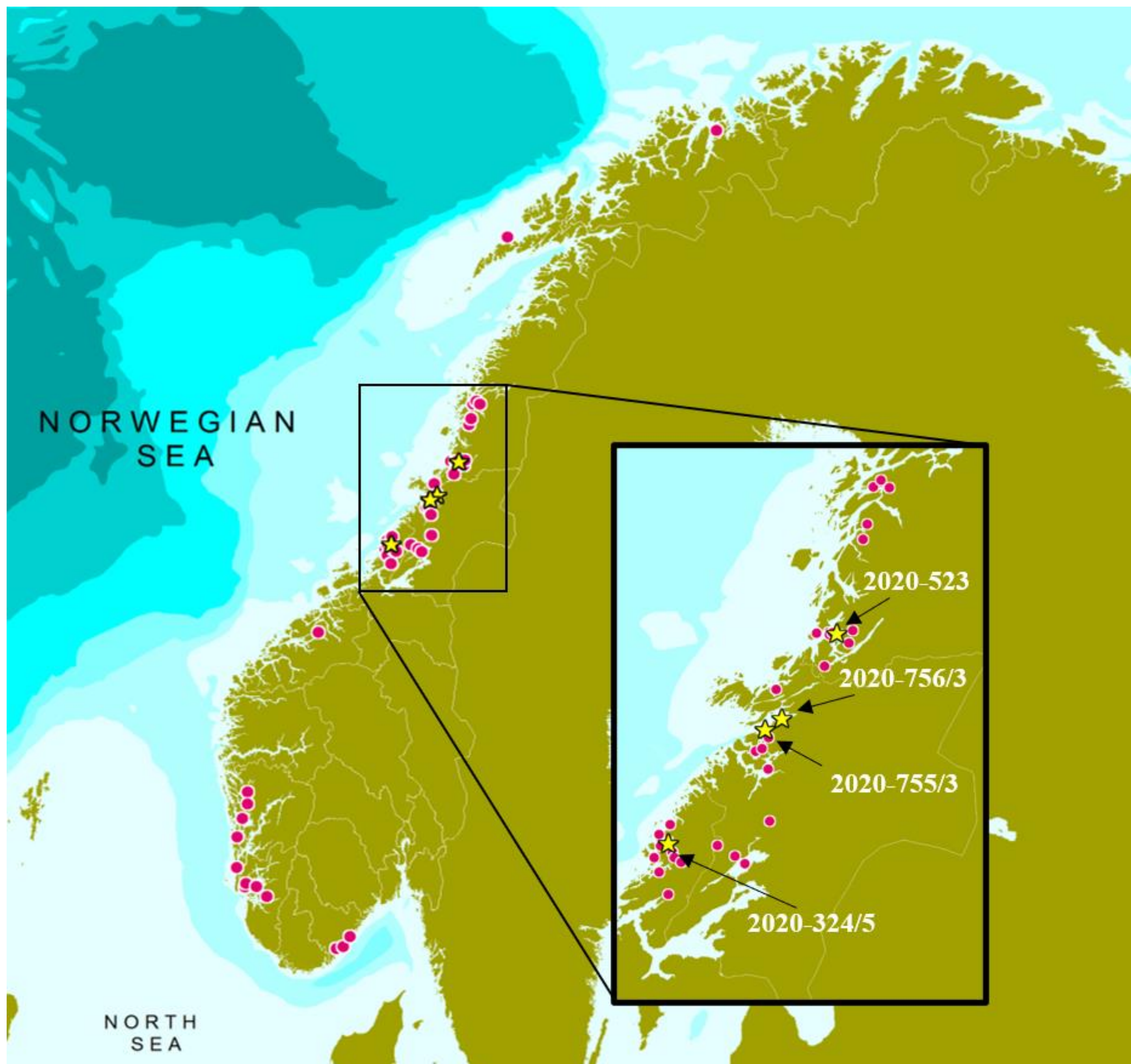

Figure 3.3: Map showing locations where antimicrobial resistant enterococci were found. Isolates from Nordland and Trøndelag are enlarged in bottom right corner, and the locations of the four sequenced *E. faecium* isolates are indicated with stars.

For the tested antimicrobial agents, 30 different phenotypic resistance combinations were observed among the 88 resistant isolates (Fig. 3.4). The most common combination was seen in 12 isolates that were resistant to the antimicrobial agents ciprofloxacin and norfloxacin. Of all the resistant isolates, 39 (44%) were resistant to only one antimicrobial agent and 24 (27%) to three or more.

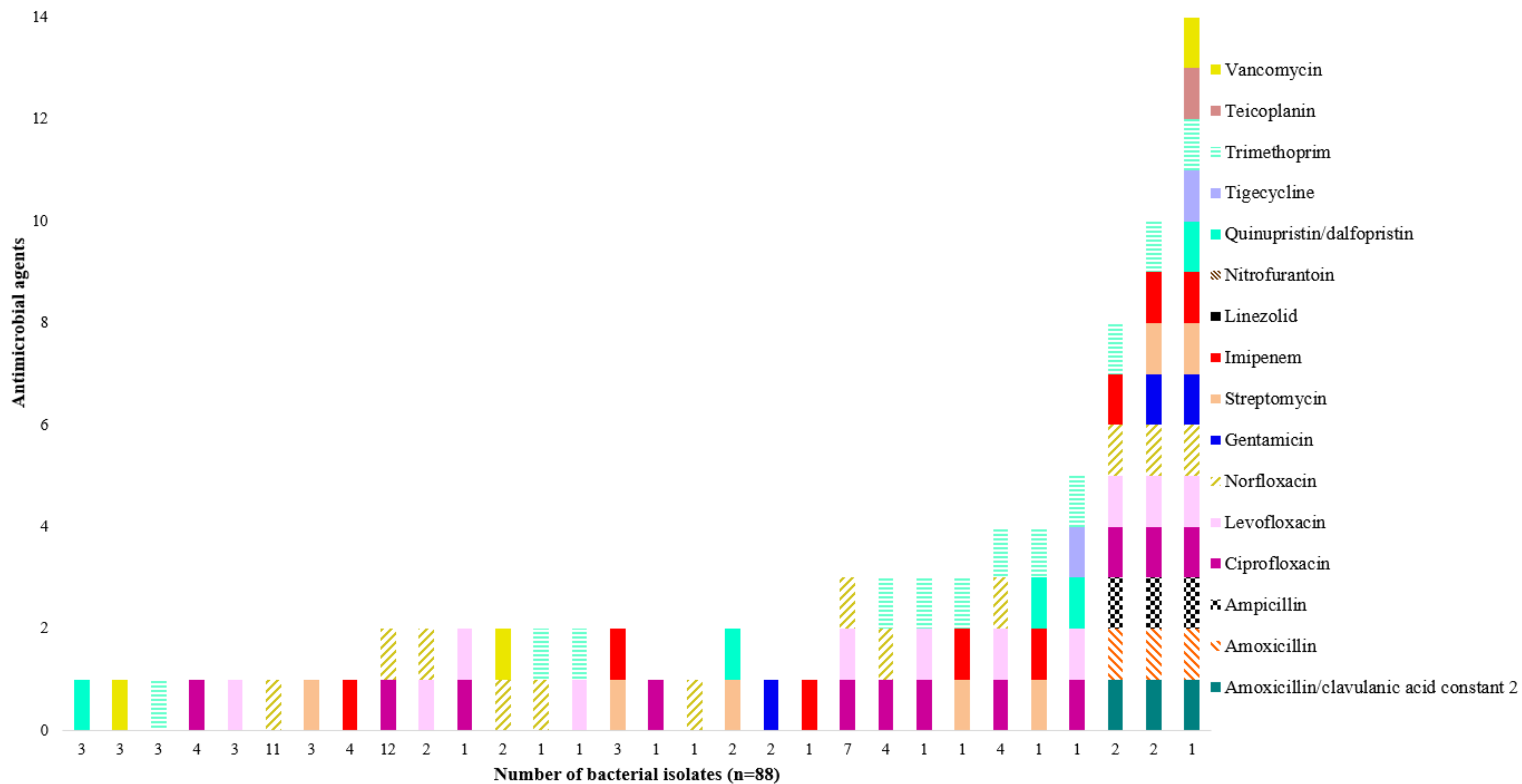

Figure 3.4. Number of bacterial isolates (n=88) showing resistance to the antimicrobial agents based on the EUCAST Clinical Breakpoint Tables v.11.

3.5 Heavy metal susceptibility testing

3.5.1 Broth dilution method unsuccessful in testing for HMR

Testing for heavy metal susceptibility by the broth dilution method was unsuccessful. Growth in the wells was poor and it was challenging to determine growth, especially separating growth from no growth. Further, when using spectrophotometric methods, none of the applied wavelength filters were able to differentiate between actual bacterial growth and the pigmentation from the copper solution. Wells with bacterial growth and wells containing only copper solution had similar absorbance patterns (Fig. 3.5), which made it impossible to calculate the bacterial density of each well.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.642 A	0.471 A	0.500 A	0.515 A	0.504 A	0.504 A	0.466 A	0.527 A	0.468 A	0.512 A	0.490 A	0.685 A
B	0.428 A	0.440 A	0.439 A	0.468 A	0.441 A	0.444 A	0.417 A	0.455 A	0.470 A	0.444 A	0.424 A	0.576 A
C	0.435 A	0.522 A	0.511 A	0.494 A	0.480 A	0.489 A	0.489 A	0.518 A	0.374 A	0.532 A	0.470 A	0.556 A
D	0.453 A	0.465 A	0.478 A	0.495 A	0.487 A	0.494 A	0.482 A	0.467 A	0.535 A	0.486 A	0.477 A	0.512 A
E	0.498 A	0.517 A	0.516 A	0.507 A	0.527 A	0.527 A	0.522 A	0.485 A	0.539 A	0.567 A	0.565 A	0.546 A
F	0.463 A	0.486 A	0.491 A	0.470 A	0.500 A	0.485 A	0.486 A	0.507 A	0.467 A	0.463 A	0.472 A	0.559 A
G	0.476 A	0.488 A	0.489 A	0.483 A	0.487 A	0.439 A	0.512 A	0.543 A	0.501 A	0.496 A	0.485 A	0.512 A
H	0.322 A	0.412 A	0.388 A	0.402 A	0.411 A	0.414 A	0.423 A	0.426 A	0.424 A	0.433 A	0.452 A	0.305 A

Figure 3.5: Spectrophotometric methods were tested to measure the bacterial density of enterococci in the wells. Wells contained bacterial suspensions in copper sulphate in different concentrations and Mueller Hinton Broth. Bacterial suspensions were applied in rows A to G, and copper sulphate solution in columns 1 to 11, from high to low concentration: 80 mM, 72 mM, 64 mM, 56 mM, 48 mM, 40 mM, 32 mM, 24 mM, 16 mM, 8 mM and 4 mM.

3.5.2 Agar dilution method successful for the detection of HMR in enterococci

All 480 isolates were tested for heavy metal susceptibility. Among 478 of the isolates, 43 isolates expressed resistance to heavy metals. Eight isolates (2 %) showed resistance to copper, 35 (8 %) to cadmium and none showed resistance to zinc. One isolate each of *E. faecium* and *E. faecalis* was resistant to both copper and cadmium. The species with the highest number of copper resistant isolates was *E. faecium* (Table 3.6). For the distribution of the MIC values for all tested species of *Enterococcus,* see Appendix 8.4. The 43 HMR isolates comprised *E. faecium* (40%), *E. faecalis* (37%), *E. hirae* (16%) and *E. casseliflavus* (7%).

Table 3.6. The distribution of MIC values for 245 *E. faecium* isolates for each heavy metal. Dark cells indicate isolates with MICs above the ECOFF values. White cells indicate the concentrations included for the three metals.

E. faecium	Distribution (n) of MIC values (mg/L)												
	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	Total
Copper								1	198	40	6		245
Zinc									1	212	32		245
Cadmium	1	20	60	144	8	9		2	1				245

Twelve isolates (3%) were resistant to both heavy metals and antimicrobials, and consisted of the species *E. faecium*, *E. faecalis* and *E. casseliflavus* (Table 3.7).

Table 3.7. An overview of the 12 isolates that expressed antimicrobial and HMR and from which sample species they were isolated.

Isolate No.	Species	AMR MIC (mg/L)	HMR MIC (mg/L)	Sample
2016-687/1	*E. faecium*	Norfloxacin MIC 16	Cadmium MIC 1	Blue mussel
2020-522/1	*E. casseliflavus*	AMOCLA MIC 32 Amoxicillin MIC 32 Ampicillin MIC 32 Ciprofloxacin MIC 16 Levofloxacin MIC 16 Norfloxacin MIC 16 Imipenem MIC 16 Trimethoprim MIC 4	Copper MIC 32	Blue mussel
2016-1216/1	*E. faecalis*	Ciprofloxacin MIC 16	Cadmium MIC 16	Blue mussel
2016-374/1	*E. faecium*	Streptomycin MIC 1024	Copper MIC 32	European flat oyster
2016-813/1	*E. faecalis*	Streptomycin MIC 1024	Cadmium MIC 1	Blue mussel
2016-1290/1	*E. faecium*	Ciprofloxacin MIC 16 Levofloxacin MIC 16 Trimethoprim MIC 4	Cadmium MIC 4	Blue mussel
2016-644/1	*E. faecium*	Imipenem MIC 8	Cadmium MIC 1	Blue mussel
2016-1030/1	*E. faecalis*	Gentamicin MIC 256	Cadmium MIC 4	Blue mussel
2016-1030/2	*E. faecalis*	Gentamicin MIC 256	Cadmium MIC 4	Blue mussel
2016-678/1	*E. faecium*	Ciprofloxacin MIC 8 Norfloxacin MIC 16	Cadmium MIC 1	Blue mussel
2016-883/1	*E. faecium*	Ciprofloxacin MIC 8 Norfloxacin MIC 16	Cadmium MIC 1	Blue mussel
2020-983/2	*E. faecalis*	Streptomycin MIC 1024	Cadmium MIC 8	Blue mussel

AMR=Antimicrobial resistance, MIC=Minimum Inhibitory Concentration, AMOCLA=Amoxicillin/Clavulanic acid

3.6 Whole genome sequencing

3.6.1 WGS quality, phylogenetic analyses and detection of antimicrobial resistance genes

Four antimicrobial resistant *E. faecium* isolates from different samples were subjected to whole genome sequencing (Table 3.8). The WGS showed good quality: All WGS had less than 300 contigs after assembly and a coverage above 100. Genome sizes were between 2,7 to 2,9 mega base pairs (Mb) which is within the normal range for *E. faecium* genomes. Multi Locus Sequence Typing (MLST) and core genome Multi Locus Sequence Typing (cgMLST) identified isolates 2020-755/3 and 2020-756/3 as sequence type (ST) 117 and cluster type (CT) 2505, and isolate 2020-523/1 as ST80 with an unknown CT (Table 3.7). Isolate 2020-324/5 had the novel ST1484 and an unknown CT. Core genome analyses by two different methods showed that the two ST117 isolates cluster together while the other isolates were further apart (Fig. 3.6 and Fig. 3.7). The ST80 isolate clusters with clinical *E. faecium* strains while the ST1484 isolate clusters with commensal strains (Fig. 3.6). Several genes coding for antimicrobial resistance were also found, and the phenotypic resistance data for gentamicin and ciprofloxacin resistance was supported by these findings (Table 3.10).

Table 3.8. Source, phenotypic resistance, ST and CT of the *E. faecium* were subjected to WGS.

Isolate	Species	From bivalve	Phenotypic resistance	MLST	CT
2020-755/3	*E. faecium*	Blue mussel	MIC 32 mg/L Ampicillin, MIC 256 mg/L Gentamicin	117	2505
2020-756/3	*E. faecium*	Blue mussel	MIC 32 mg/L Ampicillin, MIC 256 mg/L Gentamicin	117	2505
2020-523/1	*E. faecium*	Blue mussel	MIC 32 mg/L Ampicillin	80	Unknown
2020-324/5	*E. faecium*	Blue mussel	MIC 8 mg/L Q/D	1484	Unknown

Q/D=Quinupristin/dalfopristin

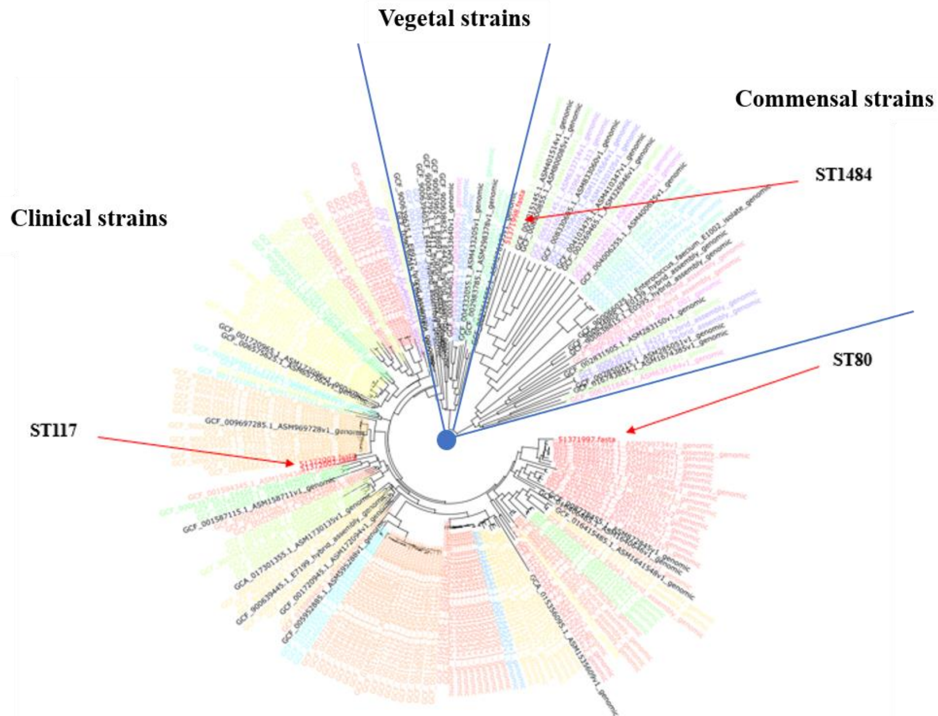

Figure 3.6: Global Parsnp tree of *E. faecium* genomes with the location of the marine isolates (red) and their three STs indicated. The blue lines divide the tree into different sections. The branch length of the novel ST1484 from isolate 2020-324/5 compared to the commensal strains suggests it is very different from genomes that have been sequenced before.

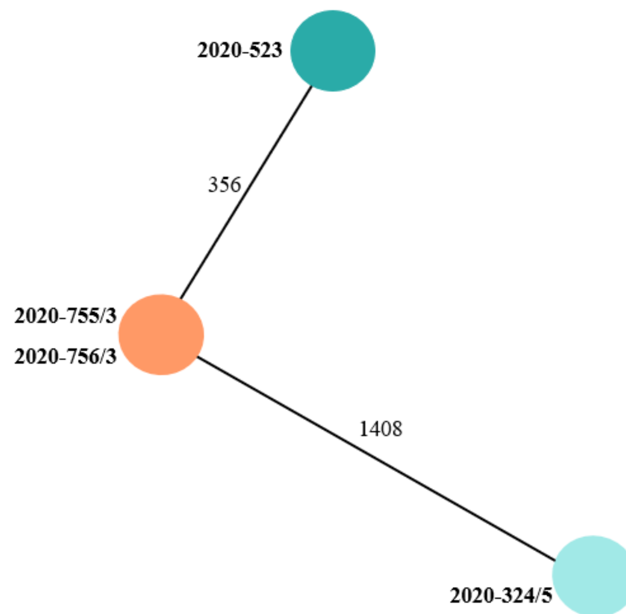

Figure 3.7: SeqSphere cg(MLST) clustering of the four *E. faecium*. Numbers between clusters indicate differences in Single nucleopeptide polymorphisms (SNPs) between each isolate. The novel ST1484 from isolate 2020-324/5 shows the largest number of SNP differences compared to the other isolates. Isolates 2020-755/3 and 2020-756/3 share the same colour to indicate that they are very similar.

3.6.2 Detection of HMR genes

The four genome-sequenced *E. faecium* isolates derived from four different samples that showing phenotypic resistance to antimicrobials as well as the presence of several antimicrobial- and HMR genes (Table 3.9). Isolates 2020-755/3, 2020-756/3 and 2020-324/5 had two HMR genes and isolate 2020-523/1 had three (Table 3.5).

Table 3.9: An overview of the hits from the BacMet database for HMR genes found in the four resistant genome-sequenced *E. faecium*.

Gene	Function	Source bacteria	2020-755/3	2020-756/3	2020-523/1	2020-324/5
lde	Multidrug transporter MdtG	*Listeria monocytogenes*				x
chtS	Sensor histidin kinase	*Enterococcus*	x	x	x	x
copA	Copper-translocating P-type ATPase	*Enterococcus*	x	x	x	
gadC/xasA	Glutamate gamma-aminobutyrate antiporter	*Enterococcus*			x	

Table 3.10. Antimicrobial- and HMR genes and observed phenotypic resistance of the four resistant *E. faecium* subjected to WGS. All four isolates were susceptible to the heavy metals copper and cadmium.

	ST	AG	LP	ML	PM	Q	ST	TC	TP	HMRG	Phe. Res
2020-755/3	117	*ant(6)-Ia.* *aph(3')-IIIa* *aac(6')-I* [1]		*msr(C)* *erm(B)*	*eat(A)*	*gyrA*_S83Y *parC*_S80I	*sat4*		*dfrG*	*copA* *chtS*	AMP, GENT, CIP
2020-756/3	117	*ant(6)-Ia.* *aph(3')-IIIa* *aac(6')-I*		*msr(C)* *erm(B)*	*eat(A)*	*gyrA*_S83Y *parC*_S80I	*sat4*		*dfrG*	*copA* *chtS*	AMP, GENT, CIP
2020-523/1	80	*aac(6')-I*	*liaS*_T120A (DAPTOMYCIN) *liaR*_W73C (DAPTOMYCIN)	*msr(C)*	*eat(A)*	*gyrA*_S83Y. *parC*_S80I		*tet(L)*	*dfrG*	*copA* *chtS* *gadC/xasA*	AMP, CIP
2020-324/5	1484	*aac(6')-I*		*msr(C)*						*chtS* *lde*	Q/D

AG=Aminoglycoside, LP=Lipopeptide, ML=Macrolide, PM=Pleuromutilin, Q=Quinolone, ST=Streptothricin, TC=Tetracycline, TP=Trimethoprim, Phe. Res=Phenotypic Resistance.

[1]*: The gene aac(6')-I is intrinsic to* E. faecium *and confers low-level aminoglycoside resistance.*

4. Discussion

This study aimed to examine the prevalence of bacteria belonging to the genus *Enterococcus* in the Norwegian marine environment by using bivalve molluscs as sentinel tools. The possible influence of seasons and geographical location could affect the prevalence. Further, by characterising antimicrobial and HMR patterns and their associated genes, the possible co-selection of these traits was examined. The origin of the *Enterococcus* spp. isolates was examined and applicability of enterococci as indicators of faecal pollution was discussed.

4.1 Prevalence of enterococci in marine bivalves

The results from this study revealed a 60% prevalence (± 4.6%, 95% CI) of enterococci in the bivalve samples and that certain bivalves periodically accumulate high levels reaching a concentration up to 3500 MPN/100g, as found in one sample of blue mussel. The majority of the samples, however, had low levels and 98% of the samples were below 1000 MPN/100g which is the limit of detection of the standardised method for enterococci in foodstuffs (NMKL, 2011). From the bivalve samples (n=473), 480 isolates were obtained. The 480 isolates represented ten enterococcal species, roughly 17% of the known enterococcal species identified as of today. Most of the isolates were *E. faecium* and *E. faecalis*, which are also the most abundant in humans and animals (Lebreton et al., 2014).

These findings showed that many of the well-known enterococcal species can be found in the marine environment but in rather low levels, which is positive for the consumption of popular seafoods such as blue mussel, scallop, or oyster.

Half of the samples were from Trøndelag, which was expected as this county is the main production area for farmed blue mussel in Norway. Among the samples from Trøndelag, 96% were from blue mussel, which was also the dominating bivalve species in all other counties. In the counties located on the western coast of Norway, Rogaland and Vestland, the number of great scallop and European flat oyster samples were almost identical to that of blue mussel.

The highest prevalence of bivalve samples with enterococci was seen in Agder, where 14 of the 18 samples (78%) contained enterococci (Table 3.5). For the remaining counties, the sample prevalence was between 56 and 67%.

4.2 Effects of seasonality on the prevalence of enterococci

For enterococci, there was a significant difference in the MPN/100g during the summer months compared to spring and autumn months, where sampling through the summer gave an increased number of samples with higher concentrations. Despite this, the highest individual numbers were observed during autumn. The observed difference could be linked to the melting of ice during spring that increases the run-off from land through rivers and sewage systems (Lunestad et al., 2016). It could also be that warmer sea surface temperatures improve the growth conditions for the marine enterococci (Vezzulli et al., 2016).

4.3 Antimicrobial and heavy metal resistance among bacterial isolates

4.3.1 Prevalence of antimicrobial resistant enterococci

Among the 247 *E. faecium* isolates, 76% were resistant to one or more antimicrobials (Table 3.3). Resistance to streptogramins was found in four isolates (Table 3.4), of which one was among the four isolates subjected to WGS. Our findings are in accordance with previous studies suggesting that resistance to the streptogramin Q/D in *E. faecium* is rare (Shaw et al., 2018) and was also in agreement with the declining usage of streptogramins for humans in Norway (NORM/NORM-VET, 2019). In a study by Donabedian et al. where more than 300 Q/D resistant *E. faecium* were isolated from humans, animals and meat in the US, almost no genes known to confer resistance to Q/D were found, suggesting that there may be other not yet found mechanisms of Q/D resistance in *E. faecium* (Donabedian et al., 2006). Among the *E. faecium* isolates in the current study, 2.5% were multidrug resistant. The two most resistant isolates were those who also belonged to the hospital-associated sequence types (isolates 2020-755/3 and 2020-756/3) and showed resistance to penicillins, aminoglycosides, quinolones, and imipenem.

The number of antimicrobial resistant *E. faecalis* isolates was only 8%, which was low compared to that of *E. faecium*. Resistance was seen towards fluoroquinolones, aminoglycosides, and imipenem. The observed resistance among the other detected enterococci varied between the species, where three each of *E. avium*, *E. casseliflavus*, *E. thailandicus,* two *Enterococcus* spp. and one each of *E. durans, E. hirae* and *E. villorum* were found to be resistant. These species have all been associated with human disease except *E. casseliflavus* which is associated with soil and *E. villorum* to pig. All the isolates belonging to *E. gallinarum*

were resistant to at least one antibiotic, and one isolate expressed resistance to eight different antimicrobial classes, but not to any of the heavy metals. The expression of resistance to such a high number of antimicrobials was not surprising knowing that this species is intrinsically resistant to several antimicrobials (Ahmed & Baptiste, 2018).

Fortunately, resistance to the glycopeptide vancomycin and the oxazolidinone linezolid was not observed in any of the enterococcal species. The absence of vancomycin resistant *E. faecium* or *E. faecalis* in all the examined samples is positive and indicates little dissemination of VRE from hospital sewage in the locations where the bivalves were sampled (Oravcova et al., 2017). This reflects the restrictive use of glycopeptides in Norway and that VRE is rare in human clinical enterococcal isolates (NORM/NORM-VET, 2019). Linezolid resistance in enterococci is becoming more frequent, also in Norway, but the global prevalence is still below 1% (Bender et al., 2018).

The prevalence of antimicrobial resistant enterococci differed in each county and was highest in Agder (56%), and lower in Nordland (24%), Trøndelag (18%), Vestland (11%), Rogaland (11%) and Troms & Finnmark (17%). Agder county lies in the south of Norway with a warmer climate and a long coastline bordering to the Skagerrak and North Sea. It is the sixth most densely populated county in Norway (Statistisk Sentralbyrå, 2020) and has three large hospitals located in the coastal cities Kristiansand, Arendal and Flekkefjord. Interestingly, Agder is also the highest antimicrobial-consuming county in Norway, with 15 Defined Daily Doses (DDD) per 1000 inhabitants per day (NORM/NORM-VET, 2019). However, only 18 samples represented this county, and more data would be needed to investigate a possible link. Half of the bivalve samples came from Trøndelag, and here the prevalence of antimicrobial resistance per sample was less than half of what was seen in Agder.

4.3.2 Antimicrobial resistance genes

Interestingly, quinolone resistance was seen in three of the four sequenced isolates. This associated well with the phenotypic ciprofloxacin resistance in isolates 2020-755/3, 2020-756/3 and 2020-523/1. Quinolone resistance mutations are typically associated with hospital adapted clones and are also seen in clade A1 (Leavis et al., 2006) which is known for containing clinical strains. The isolates identified as ST117 and ST80 (isolates 2020-755/3, 2020-756/3 and 2020-523/1) have the quinolone resistance mutations in genes *gyrA_S83Y* and *parC_S80I*, which fits well with previous studies connecting hospital adapted clones to quinolone resistance. Both genes *gyrA_S83Y* and *parC_S801* are essential in enterococci, coding for the

enzymes gyrase and topoisomerase, respectively (Petersen & Jensen, 2004). When a certain number of mutations occur in these genes, resistance to quinolones is developed and the synthesis of these enzymes is inhibited. Quinolones are known as broad-spectrum antimicrobials that have a large eco-shadow. Antimicrobials with a large eco-shadow are likely to induce more unwanted side-effects in the patient and affect many other sites in the body than only the target site, such as the patient's healthy microbial flora in the gut (NLHB, 2021). Disrupting the natural balance of the microbes in the gut can lead to over-growth by certain species and facilitate the development of antimicrobial resistant strains (NLHB, 2021).

Genetic analyses showed that the gene *aac(6')-l* (Table 3.10) which codes for aminoglycoside resistance was present in all four isolates. As this gene is intrinsic for *E. faecium*, they will naturally express low-level resistance towards aminoglycosides such as gentamicin. In order for *E. faecium* to express high-level resistance to aminoglycosides, more than one gene coding for aminoglycoside resistance has to be present, as seen in isolates 2020-755/3 and 2020-756/3 where the three genes *ant(6)-Ia, aph(3')-IIIa* and *aac(6')-I* were found. These isolates had a MIC for gentamicin of 256 mg/L.

Resistance to lipopeptide, macrolide, pleuromutilin, tetracycline and trimethoprim (MIC>1 mg/L, non-wildtype) was also observed among the four resistant *E. faecium*.

4.3.3 Heavy metal resistance

The species revealing heavy metal resistance included *E. faecium* (40%), *E. faecalis* (37%), *E. hirae* (16%) and *E. casseliflavus* (7%). Among seventeen heavy metal resistant *E. faecium* isolates, 35% were found resistant to copper and 65% were resistant to cadmium. Cadmium is known to appear in soil and is one of the most toxic metals in inorganic fertilising products, but there is still little known about the potential release of cadmium resistance genes to the environment via fertilising products (Wastson et al., 2019). Six of the heavy metal resistant *E. faecium* were also resistant to the antimicrobials ciprofloxacin (50%), norfloxacin (50%) and levofloxacin, streptomycin, or imipenem (17%).

All heavy metal resistant *E. faecalis* isolates were resistant to cadmium and only one of these expressed resistance to copper (MIC 40 mg/L). The copper resistant isolate was not resistant to any antimicrobials.

Although no isolates were resistant to zinc based on the MIC breakpoint value of 64 mg/L, MIC values seemed to differ between species. In *E. faecium*, *E. hirae* and *E. durans* isolates, MIC 16 mg/L was the most common value (in 87%, 81% and 88% of the isolates, respectively)

and in *E. faecalis* MIC 32 mg/L was the most common (in 95% of the isolates). It would seem that *E. faecalis* in general has a higher tolerance to zinc than the other enterococcal species. All isolates could grow in the presence of zinc, which raises the question whether all isolates in fact were resistant to zinc but that the breakpoint value was set too high in the ECOFFinder. Applying a breakpoint value based on MIC values is mostly done for pharmaceutical drugs such as antimicrobials, but not so commonly for heavy metals. Heavy metals are also molecularly very different from antimicrobials – thus MIC values between antimicrobials and heavy metals should be compared with caution.

None of the four sequenced *E. faecium* expressed phenotypic resistance to heavy metals when tested on agar. Nevertheless, it was not unexpected to detect genes involved in heavy metal resistance as they are needed to regulate metal homeostasis in the bacteria. When running these isolated through the BacMet database, four genes involved in HMR (Table 3.4) were revealed: *lde*, *chtS*, *copA* and *gadC/xasA*. The *lde* gene was found in isolate 2020-324/5 which was the only isolate not associated to the hospital environment. This gene encodes a transmembrane efflux pump previously found in *Listeria monocytogenes*, but since enterococci and *L. monocytogenes* both are Gram-positive and thus share similar membranous structures, it is likely that the gene encodes similar proteins in both species. Efflux pumps are important in bacteria for releasing toxic compounds, such as metals or antimicrobials, from the cell out in the environment (Webber & Piddock, 2003), which could naturally explain the presence of this gene in any bacterium. It is unsure why only one of the isolates has this gene, and the others do not.

The sensor histidine kinase coding gene *chtS* was found in all four isolates. Histidine kinases are usually trans-membranous proteins involved in transferring signals across the cell membrane (Wolanin et al., 2002). Interestingly, in a study from the Netherlands a two-component system between *chtS* and another gene *chtR* was found to contribute to resistance towards the disinfectant chlorhexidine in *E. faecium* (Prieto et al., 2017), but as *chtR* was not found in this study, the four isolates may not be resistant to chlorhexidine. Testing the isolates for phenotypic chlorhexidine resistance would have to be done before confirming resistance to this disinfectant.

The *copA* gene (Table 3.10) is part of the *cop* operon known to regulate copper homeostasis in *E. hirae* and other enterococcal species (Hasman & Aarestrup, 2002). This gene codes for a membranous ATPase protein CopA responsible for transporting copper into the cell when

copper levels are low. Most likely this gene is naturally present in enterococci and has a necessary function in regulating the copper levels within the cell. Other genes are known to confer resistance to copper in enterococci, such as the *tcrB* gene together with the *cop* operon but these were not detected. As the *tcrB* gene was not found in these four isolates, they might not have come from environments where copper levels were high, such as the intestine of pigs, broilers, or calves. As described by Hasman & Aarestrup (Hasman & Aarestrup, 2002), these species commonly receive copper added to their feed.

Only the isolate 2020-523/1 contained the *gadC/xasA* gene. This gene encodes a glutamate/gamma-aminobutyrate antiporter needed to exchange glutamate from the extracellular environment with gamma-aminobutyric acid (GABA) from the intracellular environment when pH is low, which is important for example in bacteria that exist in the acidic environment of the stomach. The presence of this gene could indicate that this isolate is adapted to a very acidic environment. There is still little information available about the direct function of the *lde*, *chtS* and *gadC/xasA* genes in relation to heavy metal resistance in enterococci.

4.3.4 Co-selection of antimicrobial and heavy metal resistance

In total 12 isolates (2.5%) were resistant to both antimicrobials and heavy metals. These comprised 14 *E. faecalis*, six *E. faecium*, and one *E. casseliflavus*. Previous studies report a link between copper and vancomycin resistance particularly in *E. faecium* (Ahmed & Baptiste, 2018; Johnsen et al., 2005), but in our study vancomycin resistance was only found in *E. gallinarum* and *E. casseliflavus* (where it is intrinsic), and none of those isolates were resistant to heavy metals. The low number of vancomycin resistant isolates in the current study could be explained by the restrictive use of antimicrobials in Norway where it is considered a last resort antimicrobial. If vancomycin resistant *E. faecium* had been detected, finding genes conferring HMR might have been more likely. Low levels of antimicrobial and heavy metal resistance in the enterococci make it more challenging to detect the presumptive co-selection of genes conferring resistance to these traits. Nevertheless, it is positive that such low levels are detected as this could indicate that the co-transfer of antimicrobial and heavy metal resistance genes among marine enterococci is rare.

4.4 The origin of marine enterococci

Within the bivalve samples, the composition of *Enterococcus* species varied. Examining which species occur together could say something about the origin of the enterococci and what kind of pollution that could have been in the area. The most frequently observed combination of

enterococcal species was *E. faecium* and *E. hirae* occurring 18 samples. The presence of *E. faecium* and *E. hirae* is possibly due to faecal pollution as they are both known to be common in human and animal faeces, and *E. hirae* also in plants and insects (Table 1.1) (Lebreton et al., 2014). Other identified species, such as *E. villorum* and *E. casseliflavus* have been associated with pigs and soil, respectively. This shows that the enterococci in bivalves could originate from multiple sources.

Among the four selected *E. faecium* isolates resistant to antimicrobials, three were ampicillin-resistant which is typical for human clinical *E. faecium* isolates (Leavis et al., 2006; NORM/NORM-VET, 2019). The fourth isolate that showed susceptibility to ampicillin and resistance to Q/D may represent enterococcal isolates found in the marine environment that are not deriving from the hospital associated environment. Also, the general low occurrence of ampicillin resistance among the total *E. faecium* isolates (n=247) may indicate another origin than human for these because ampicillin resistance is highly linked to clinical *E. faecium*.

WGS revealed three different sequence types: ST117, ST80 and ST1484. ST117 and ST80 that were ampicillin-resistant are well-known clones from human clinical environments (Ahmed & Baptiste, 2018; Shaw et al., 2018). The multidrug-resistant ST117 is spread globally and has been found increasingly from clinical isolates especially in European health institutions (Ahmed & Baptiste, 2018). The ST1484 clusters with the commensal *E. faecium* in the global tree (Fig. 3.4) However, the origin of the ST1484 is still uncertain as this sequence type was registered for the first time in the MLST database after sequencing at K-res.

It is likely that the isolates with sequence types 117 and 80 derive from clinical institutions and have reached the ocean through sewage systems. Several other resistance genes found in the sequence types 117 and 80 (Table 3.6) are more common in clade A1 which is associated with clinical isolates (Ahmed & Baptiste, 2018). All four sequenced isolates came from farmed blue mussel samples in Trøndelag (isolate 2020-755/3, 2020-756/3 and 2020-324/5) and Nordland (isolate 2020-523/1). Isolates 2020-755/3 and 2020-756/3 were sampled only 10 kilometres apart and at the same day, and they both had the same sequence types (Fig. 3.7) suggesting they are closely related. Only five kilometres apart from the sampling site of isolate 2020-756/3 lies a sewerage installation and outlet (Miljøstatus, 2020), which could be the source of both 2020-755/3 and 2020-756/3. It is unsure whether these two isolates share the same origin or came from this exact sewerage installation, this would depend on the water currents connecting these three sites. Isolate 2020-755/3 was collected in the Namsen Fjord which is defined as a

national salmon fjord, meaning that there are specific criteria and stricter guidelines required by law to keeping this fjord clean (MD, 2007), implying that pollution in this area might be lower than in other areas and that enterococci in this area must have come via water currents or wild animals.

4.5 Enterococci as indicators of faecal pollution

Using the presence of *E. coli* as an indication of faecal pollution is a common practice globally in safety analyses of food, recreational waters, drinking water and for testing hygiene. The National Norwegian Surveillance Program for the Production of Marine Bivalves uses the MPN method for the detection of *E. coli* in bivalve molluscs to assess the degree of faecal pollution in the sampling area. In this study, we also evaluated the possible correlation between the *E. coli* and enterococci MPN numbers in samples from 2019 and 2020. There was no apparent correlation between the MPN numbers, meaning that samples containing high/low levels of *E. coli* not necessarily contain high/low levels of enterococci, and vice versa. This raises the question of whether the presence of enterococci can even be related to faecal contamination, implying that the enterococci could come from other sources that are still not identified. The isolation of *E. coli,* as used in the MPN method in the bivalve surveillance program, would therefore not be useful for giving an estimate of enterococcal pollution in the sample. This was demonstrated in sample 2020-989 where 3500 MPN/100g enterococci and <18 MPN/100g *E. coli* were found.

It would be natural to assume that an area with faecal pollution would contain both *E. coli* and enterococci, they co-exist in the intestine of humans and animals, thus bivalve molluscs should filter both. This raises the question of how there can be few enterococci in samples with high levels of *E. coli*? In samples with high levels of enterococci but few *E. coli*, an explanation can be that *E. coli* already are digested by the bivalve mollusc or otherwise dead, as *E. coli* have a much shorter lifespan than enterococci in seawater. Two factors seem to give opposing effects on the apparent lack of correlation between *E. coli* and enterococci. One is the reported differences of prevalence in the faecal material depending on its origin. As reported by Havelaar et al. (Havelaar et al., 1986), the faecal material from pig, chicken, dog, cow, horse, sheep and calf, have a ratio of thermotolerant coliforms to enterococci of 30 to 1, whereas the corresponding ratio for human faeces were reported to be 500 to 1. On the other hand, the environmental survival of enterococci is expected to be substantially higher than for *E. coli*.

Applying enterococci as indicators of faecal pollution could supplement existing methods that screen for *E. coli*, especially given that the MPN numbers for *E. coli* and enterococci were not correlated. If they had been correlated, testing for both would be unnecessary, but as this study demonstrated, a seafood sample with no *E. coli* could still contain enterococci holding a concentration above the limit of detection (<18 MPN/100g) which would not be recommended for consumption. Although the origins of marine enterococci are not yet fully uncovered, the found genetic similarities between clinical and marine isolates and the fact that sewage is released to the sea, are indications that marine enterococci to a large extent have faecal origins. Applying enterococci as indicators of faecal pollution could provide a better picture of the total faecal pollution in the sample if supplemented to existing methods.

The three ampicillin-resistant *E. faecium* that were sequenced had concentrations of *E. coli* MPN below the limit of detection (<18 MPN/100g), but enterococci MPN/100g values of 68 and 490 meaning that no correlation could be seen in these either. Isolates 2020-755/3 and 2020-756/3 were identified as belonging to the clinical sequence types 117 and had enterococcal MPN values of 68 MPN/100g. As mentioned, these were found only 10 kilometres apart. The high concentrations of enterococci in these samples could be explained by recent faecal pollution or longer ago. Given that the three ampicillin-resistant *E. faecium* were typically hospital-associated, one could think that detection of ampicillin-resistant *E. faecium* in itself is an indicator for faecal pollution. In humans, ampicillin-resistance is very high with more than 80% of all *E. faecium* being resistant to ampicillin. A Danish study on pets showed that healthy dogs and cats are carriers of the human hospital-adapted CC17 ampicillin-resistant *E. faecium* (Damborg et al., 2009), contributing to the dissemination of these strains in the community. Interestingly, a Portuguese study of faecal samples of 77 wild animals in Portugal found zero ampicillin-resistant *E. faecium* (45 isolates), the wild animals constituted owls, birds of prey, foxes, rabbits, European genets, wildcats, salamanders, storks, magpies, deer, vipers, otters, wolves, mouflons, badgers, partridges, hedgehogs, pigeons, ferrets, quails and boars (Poeta et al., 2005). These findings imply that the detection of ampicillin-resistant *E. faecium* can be linked to faecal pollution from humans or their pets, and not necessarily wild animals.

4.6 Methodological considerations

Knowing that the main production areas for farmed blue mussel are located in Trøndelag and Helgelandskysten (Duinker et al., 2020), and with half of all the samples coming from this area it was expected that a higher number of isolates would be found here. A more even geographical distribution of samples could have contributed to better comparing the presence of enterococci from different areas along the coast of Norway. There are for example no samples from around the capital of Norway, Oslo (in Viken county). It would have been interesting to include MPN numbers from bivalves sampled in these coastal areas.

The principle of the MPN method is simple but has some disadvantages. It is time consuming, requires large volumes of media to be prepared in advance, and the colour verification step can sometimes be misleading (Fig. 4.1). Most enterococcal species grew well on *Enterococcus* agar, Luria Bertani agar and Mueller Hinton agar, except *E. hirae* and *E. avium* that were very small and pale. From the *Enterococcus*-selective agar, 487 isolates were isolated at first, but analyses with MALDI-TOF MS found that seven of these belonged to other species: 3 *Pediococcus acidilactici*, 3 *Staphylococcus pasteuri* and 1 *Bacillus pumilus*, which are all Gram-positive. This meant that the agar was not entirely selective after all. These other species might have managed to grow due to insufficient heating during incubation (at 44°C), or the agar cannot distinguish between species closely related to enterococci, such as *P. acidilactici*.

Analyses with MALDI-TOF MS identified 475 of the 480 isolates belonging to ten different enterococcal species. The remaining five isolates had scores between 1.9 and 1.99 for *E. casseliflavus*, which is right below the species identification threshold of 2.0, and were therefore defined as *Enterococcus* spp. The MALDI-TOF MS method helped in making the identification of the bacterial isolates efficient and precise.

Establishing a method for the heavy metal susceptibility testing was challenging at first as the aim was to establish a microbroth dilution method similar to that used for antimicrobial susceptibility testing, which would have been a more efficient than the agar dilution method. After experiencing that the microdilution method was unsuccessful due to poor growth of the bacteria, an agar dilution method was applied. The challenge with this method was to obtain pH 7 in the agar without complex formation and precipitation. At first, sodium citrate was used to adjust the pH to ~7.0, but the amount needed to obtain the right pH was so high that the particles would not dissolve – and would often form lumps with the Mueller Hinton agar. Adjusting the pH using 4N sodium hydroxide instead gave an agar without metal precipitate.

The heavy metal containing agar precipitated during autoclaving, thus this was avoided by adding heavy metals into pre-autoclaved heated agar and heating of the mixed agar in boiling water for a few minutes and sterile controls were made to check for contamination.

5. Conclusion

This study found that enterococci were prevalent in 60 % of the 473 examined bivalves collected in 2016, 2019 and 2020 in Norway. The majority of the samples had low concentrations of enterococci. Among the 480 enterococcal isolates that were isolated, most belonged to *E. faecium*. Bivalves collected during summer months contained the highest levels of enterococci.

Resistance to antimicrobials and heavy metals was generally low and no isolates resistant to vancomycin or linezolid were found, which likely reflects the restrictive use of antibiotics in Norway. The highest prevalence of antimicrobial resistant enterococci was seen among *E. faecium* isolates (76%), and the antibiotic to which resistance was seen most frequently, were quinolones. Genomic analyses of four *E. faecium* subjected to whole genome sequencing found several genes involved in antimicrobial and heavy metal resistance. Resistance to copper and cadmium was seen, but only in a small fraction of the isolates which could imply that enterococci are exposed to low levels of heavy metals before or after entering the sea. Studying the co-selection of antimicrobial and heavy metal resistance genes was challenging due to low resistance levels. However, finding low levels of resistant enterococci is positive from an environmental and seafood safety point of view.

This study also found that resistant enterococcal isolates in bivalves were related to hospital-associated enterococci and can be found in the marine environment. These are likely introduced through sewage systems. Applying enterococci as indicators of faecal pollution could supplement existing methods for seafood safety analyses. Even though most isolates identified in this study originated from faecal pollution, other enterococcal species may enter the marine environment through other sources and would also likely be identified by the applied MPN method.

6. Further studies

When testing for heavy metal resistance, it was unsure whether none or all isolates were resistant to zinc, this matter would necessitate further research.

In this study, four *E. faecium* were sequenced and much information about their resistance genes was revealed, but more information is needed to understand the large-scale dissemination of resistance among marine enterococci and to further examine their origin. Therefore, 96 *E. faecium* have been sent to the University Hospital of Oslo for WGS and genomic analyses at K-res. These will supplement the four already sequenced isolates, and the results will hopefully contribute to get a better understanding of enterococci from the marine environment. Some of these are isolates with different resistance patterns that originated from the same sample and will hopefully say something about the strain diversity in each sample.

7. References

Agudelo Higuita, N. I., & Huycke, M. M. (2014). Enterococcal Disease, Epidemiology, and Implications for Treatment. In *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*.

Ahmed, M. O., & Baptiste, K. E. (2018). Vancomycin-Resistant Enterococci: A Review of Antimicrobial Resistance Mechanisms and Perspectives of Human and Animal Health. In *Microbial Drug Resistance*. https://doi.org/10.1089/mdr.2017.0147

Arias, C.A. and Murray, B. E. (2008). Emergence and management of drug-resistant enterococcal infections, Microbial Drug Resistance, Future Medicine. *Expert Review of Anti-Infective Therapy*, *6*(5), 637–655. http://www.futuremedicine.com/doi/abs/10.2217/ebo.12.357

Asokan, G. V., Ramadhan, T., Ahmed, E., & Sanad, H. (2019). WHO global priority pathogens list: A bibliometric analysis of medline-pubmed for knowledge mobilization to infection prevention and control practices in Bahrain. *Oman Medical Journal*, *34*(3), 184–193. https://doi.org/10.5001/omj.2019.37

Ator, L. L., Starzyk, M. J. (1976). Distribution of group D streptococci in rivers and streams. *Microbios*.

Auckland, C., Teare, L., Cooke, F., Kaufmann, M. E., Warner, M., Jones, G., Bamford, K., Ayles, H., & Johnson, A. P. (2002). Linezolid-resistant enterococci: Report of the first isolates in the United Kingdom. *Journal of Antimicrobial Chemotherapy*, *50*(5), 743–746. https://doi.org/10.1093/jac/dkf246

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., Lesin, V. M., Nikolenko, S. I., Pham, S., Prjibelski, A. D., Pyshkin, A. V., Sirotkin, A. V., Vyahhi, N., Tesler, G., Alekseyev, M. A., & Pevzner, P. A. (2012). SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. *Journal of Computational Biology*, *19*(5), 455–477. https://doi.org/10.1089/cmb.2012.0021

Ben Said, L., Hamdaoui, M., Klibi, A., Ben Slama, K., Torres, C., & Klibi, N. (2017). Diversity of species and antibiotic resistance in enterococci isolated from seafood in Tunisia. *Annals of Microbiology*, *67*(1), 135–141. https://doi.org/10.1007/s13213-016-1246-y

Bender, J. K., Cattoir, V., Hegstad, K., Sadowy, E., Coque, T. M., Westh, H., Hammerum, A. M., Schaffer, K., Burns, K., Murchan, S., Novais, C., Freitas, A. R., Peixe, L., Del Grosso, M., Pantosti, A., & Werner, G. (2018). Update on prevalence and mechanisms of resistance to linezolid, tigecycline and daptomycin in enterococci in Europe: Towards a common nomenclature. *Drug Resistance Updates*, *40*(March), 25–39. https://doi.org/10.1016/j.drup.2018.10.002

Berg, R. D. (1996). The indigenous gastrointestinal microflora. In *Trends in Microbiology* (Vol. 4, Issue 11, pp. 430–435). https://doi.org/10.1016/0966-842X(96)10057-3

Bhattacharjee, M. K. (2016). Chemistry of antibiotics and related drugs. In *Chemistry of Antibiotics and Related Drugs*. https://doi.org/10.1007/978-3-319-40746-3

Boehm, A. B., & Sassoubre, L. M. (2014). Enterococci as Indicators of Environmental Fecal

Contamination. In *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*.

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics*. https://doi.org/10.1093/bioinformatics/btu170

Byappanahalli, M. N., & Fujioka, R. (2004). Indigenous soil bacteria and low moisture may limit but allow faecal bacteria to multiply and become a minor population in tropical soils. *Water Science and Technology*, *50*(1), 27–32. https://doi.org/10.2166/wst.2004.0009

Byappanahalli, M. N., Nevers, M. B., Korajkic, A., Staley, Z. R., & Harwood, V. J. (2012). Enterococci in the Environment. *Microbiology and Molecular Biology Reviews*. https://doi.org/10.1128/mmbr.00023-12

Cabelli, V. J., Dufour, A. P., Levin, M., McCabe, L. J., & Haberman, P. W. (1979). Relationship of microbial indicators to health effects at marine bathing beaches. *American Journal of Public Health*, *69*(7), 690–696. https://doi.org/10.2105/AJPH.69.7.690

Carvalho, M. D. G. S., Steigerwalt, A. G., Morey, R. E., Shewmaker, P. L., Falsen, E., Facklam, R. R., & Teixeira, L. M. (2008). Designation of the Provisional New *Enterococcus* Species CDC PNS-E2 as *Enterococcus sanguinicola* sp. nov., Isolated from Human Blood, and Identification of a Strain Previously Named *Enterococcus* CDC PNS-E1 as *Enterococcus italicus* Fortina, Ricci, Mora, and. *Journal of Clinical Microbiology*, *46*(10), 3473–3476. https://doi.org/10.1128/JCM.00603-08

Carvalho, M. D. G. S., Steigerwalt, A. G., Morey, R. E., Shewmaker, P. L., Teixeira, L. M., & Facklam, R. R. (2004). Characterization of Three New Enterococcal Species, *Enterococcus* sp. nov. CDC PNS-E1, *Enterococcus* sp. nov. CDC PNS-E2, and *Enterococcus* sp. nov. CDC PNS-E3, Isolated from Human Clinical Specimens. *Journal of Clinical Microbiology*. https://doi.org/10.1128/JCM.42.3.1192-1198.2004

Carvalho, M. da G. S., Shewmaker, P. L., Steigerwalt, A. G., Morey, R. E., Sampson, A. J., Joyce, K., Barrett, T. J., Teixeira, L. M., & Facklam, R. R. (2006). *Enterococcus caccae* sp. nov., isolated from human stools. *International Journal of Systematic and Evolutionary Microbiology*, *56*(7), 1505–1508. https://doi.org/10.1099/ijs.0.64103-0

Cassini, A., Högberg, L. D., Plachouras, D., Quattrocchi, A., Hoxha, A., Simonsen, G. S., Colomb-Cotinat, M., Kretzschmar, M. E., Devleesschauwer, B., Cecchini, M., Ouakrim, D. A., Oliveira, T. C., Struelens, M. J., Suetens, C., Monnet, D. L., Strauss, R., Mertens, K., Struyf, T., Catry, B., … Hopkins, S. (2019). Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. *The Lancet Infectious Diseases*, *19*(1), 56–66. https://doi.org/10.1016/S1473-3099(18)30605-4

Clewell, D. B. et al. (2014). Extrachromosomal and Mobile Elements in Enterococci: Transmission, Maintenance, and Epidemiology. *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*, 1–112. http://www.ncbi.nlm.nih.gov/pubmed/24649505

Collins, D., & Farrow, E. (1984). *Enterococcus avium* nom. rev., comb. nov.; *E. casseliflavus* nom. rev., comb. nov.; *E. durans* nom. rev., comb. nov.; *E. gallinarum* comb. nov.; and *E. malodoratus* sp. nov. 220–223.

Collins, M. D., Rodrigues, U. M., Pigott, N. E., & Facklam, R. R. (1991). *Enterococcus dispar* sp. nov. a new *Enterococcus* species from human sources. *Letters in Applied Microbiology*. https://doi.org/10.1111/j.1472-765X.1991.tb00514.x

Collins MD, Facklam RR, Farrow JA, W. R. (2020). *Enterococcus raffinosus* sp. nov., *Enterococcus solitarius* sp. nov., and *Enterococcus pseudoavium* sp. nov. *Definitions*, *57*, 283–288. https://doi.org/10.32388/9avucs

Cornell University. (2009). Dairy Foods Science Notes. *Food Science*, 14853.

Courvalin, P. (2006). Vancomycin resistance in gram-positive cocci. In *Clinical Infectious Diseases*. https://doi.org/10.1086/491711

Cox, C. R., & Gilmore, M. S. (2007). Native microbial colonization of Drosophila melanogaster and its use as a model of *Enterococcus faecalis* pathogenesis. *Infection and Immunity*. https://doi.org/10.1128/IAI.01496-06

Cranford, P. J., Ward, J. E., & Shumway, S. E. (2011). Bivalve Filter Feeding: Variability and Limits of the Aquaculture Biofilter. In *Shellfish Aquaculture and the Environment*. https://doi.org/10.1002/9780470960967.ch4

Damborg, P., Top, J., Hendrickx, A. P. A., Dawson, S., Willems, R. J. L., & Guardabassi, L. (2009). Dogs are a reservoir of ampicillin-resistant *Enterococcus faecium* lineages associated with human infections. *Applied and Environmental Microbiology*. https://doi.org/10.1128/AEM.02035-08

De Been, M., Pinholt, M., Top, J., Bletz, S., Mellmann, A., Van Schaik, W., Brouwer, E., Rogers, M., Kraat, Y., Bonten, M., Corander, J., Westh, H., Harmsen, D., & Willems, R. J. L. (2015). Core genome multilocus sequence typing scheme for high-resolution typing of *Enterococcus faecium*. *Journal of Clinical Microbiology*, *53*(12), 3788–3797. https://doi.org/10.1128/JCM.01946-15

de Kraker, M. E. A., Jarlier, V., Monen, J. C. M., Heuer, O. E., van de Sande, N., & Grundmann, H. (2013). The changing epidemiology of bacteraemias in Europe: Trends from the European antimicrobial resistance surveillance system. *Clinical Microbiology and Infection*, *19*(9), 860–868. https://doi.org/10.1111/1469-0691.12028

Del Mar Lleo', M., Tafi, M. C., & Canepari, P. (1998). Nonculturable *Enterococcus faecalis* cells are metabolically active and capable of resuming active growth. *Systematic and Applied Microbiology*, *21*(3), 333–339. https://doi.org/10.1016/S0723-2020(98)80041-6

Desmarais, T. R., Solo-Gabriele, H. M., & Palmer, C. J. (2002). Influence of soil on fecal indicator organisms in a tidally influenced subtropical environment. *Applied and Environmental Microbiology*, *68*(3), 1165–1172. https://doi.org/10.1128/AEM.68.3.1165-1172.2002

Devriese, L. A. (1990). *Enterococcus columbae*, a species from pigeon intestines. *71*.

Devriese, L. A., Dutta, G. N., Farrow, J. A. E., Van de Kerckhove, A., & Phillips, B. A. (1983). *Streptococcus cecorum*, a New Species Isolated from Chickens. *International Journal of Systematic Bacteriology*, *33*(4), 772–776. https://doi.org/10.1099/00207713-33-4-772

Donabedian, S. M., Perri, M. B., Vager, D., Hershberger, E., Malani, P., Simjee, S., Chow, J., Vergis, E. N., Muder, R. R., Gay, K., Angulo, F. J., Bartlett, P., & Zervos, M. J. (2006). Quinupristin-dalfopristin resistance in *Enterococcus faecium* isolates from humans, farm

animals, and grocery store meat in the United States. *Journal of Clinical Microbiology*, *44*(9), 3361–3365. https://doi.org/10.1128/JCM.02412-05

Duinker, A., Storesund, J., Lunestad, B. T., & Sanden, M. (2020). *Nasjonalt tilsynsprogram for produksjon av skjell og andre bløtdyr*.

ECDC. (2013). *Point Prevalence Survey of Healthcare-Associated Infections and Antimicrobial Use in European Acute Care Hospitals 2011-2012*.

EUCAST. (2021). Breakpoint tables for interpretation of MICs and zone diameters. Version 11.0, 2021. *Http://Www.Eucast.Org*, 0–77. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_5.0_Breakpoint_Table_01.pdf

Farrow, J. A. E., & Collins, M. D. (1985). *Enterococcus hirae*, a new species that includes amino acid assay strain NCDO 1258 and strains causing growth depression in young chickens. *International Journal of Systematic Bacteriology*, *35*(1), 73–75. https://doi.org/10.1099/00207713-35-1-73

Feldgarden, M., Brover, V., Haft, D. H., Prasad, A. B., Slotta, D. J., Tolstoy, I., Tyson, G. H., Zhao, S., Hsu, C. H., McDermott, P. F., Tadesse, D. A., Morales, C., Simmons, M., Tillman, G., Wasilenko, J., Folster, J. P., & Klimke, W. (2019). Validating the AMRFINder tool and resistance gene database by using antimicrobial resistance genotype-phenotype correlations in a collection of isolates. *Antimicrobial Agents and Chemotherapy*. https://doi.org/10.1128/AAC.00483-19

Fiedler, S., Bender, J. K., Klare, I., Halbedel, S., Grohmann, E., Szewzyk, U., & Werner, G. (2016). Tigecycline resistance in clinical isolates of *Enterococcus faecium* is mediated by an upregulation of plasmid-encoded tetracycline determinants tet(L) and tet(M). *Journal of Antimicrobial Chemotherapy*. https://doi.org/10.1093/jac/dkv420

Figdor, D., Davies, J. K., & Sundqvist, G. (2003). Starvation survival, growth and recovery of *Enterococcus faecalis* in human serum. *Oral Microbiology and Immunology*, *18*(4), 234–239. https://doi.org/10.1034/j.1399-302X.2003.00072.x

Fiore, Elizabeth, Van Tyne, Daria, Gilmore, M. S. (2019). Pathogenicity of Enterococci. *Microbiology Spectrum*. https://doi.org/10.1128/microbiolspec.gpp3-0053-2018

Fortina, M. G., Ricci, G., Mora, D., & Manachini, P. L. (2004). Molecular analysis of artisanal Italian cheeses reveals *Enterococcus italicus* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, *54*(5), 1717–1721. https://doi.org/10.1099/ijs.0.63190-0

García-Solache, M., & Rice, L. B. (2019). The *Enterococcus*: A model of adaptability to its environment. *Clinical Microbiology Reviews*, *32*(2), 1–28. https://doi.org/10.1128/CMR.00058-18

Gaynes, R. (2017). The discovery of penicillin—new insights after more than 75 years of clinical use. *Emerging Infectious Diseases*. https://doi.org/10.3201/eid2305.161556

Ghosh, A., KuKanich, K., Brown, C. E., & Zurek, L. (2012). Resident cats in small animal veterinary hospitals carry multi-drug resistant enterococci and are likely involved in cross-contamination of the hospital environment. *Frontiers in Microbiology*, *3*(FEB), 1–14. https://doi.org/10.3389/fmicb.2012.00062

Gilmore, M. S. (2002). The Enterococci: pathogenesis, molecular biology, and antibiotic

resistance. In *ASM Press*.

Gilmore, M. S., Lebreton, F., & van Schaik, W. (2013). Genomic transition of enterococci from gut commensals to leading causes of multidrug-resistant hospital infection in the antibiotic era. In *Current Opinion in Microbiology*. https://doi.org/10.1016/j.mib.2013.01.006

Gold, H. S. (2001). Vancomycin-resistant enterococci: Mechanisms and clinical observations. *Clinical Infectious Diseases*. https://doi.org/10.1086/321815

Gomes, B. P. F. A., Pinheiro, E. T., Sousa, E. L. R., Jacinto, R. C., Zaia, A. A., Ferraz, C. C. R., & de Souza-Filho, F. J. (2006). *Enterococcus faecalis* in dental root canals detected by culture and by polymerase chain reaction analysis. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontology*. https://doi.org/10.1016/j.tripleo.2005.11.031

Gonzales, R. D., Schreckenberger, P. C., Graham, M. B., Kelkar, S., DenBesten, K., & Quinn, J. P. (2001). Infections due to vancomycin-resistant *Enterococcus faecium* resistant to linezolid. *Lancet*. https://doi.org/10.1016/S0140-6736(00)04376-2

Gurevich, A., Saveliev, V., Vyahhi, N., & Tesler, G. (2013). QUAST: Quality assessment tool for genome assemblies. *Bioinformatics*. https://doi.org/10.1093/bioinformatics/btt086

Hardie, J. M., & Whiley, R. A. (1997). Classification and overview of the genera *Streptococcus* and *Enterococcus*. In *Society for Applied Bacteriology symposium series*.

Hartke, A., Giard, J. C., Laplace, J. M., & Auffray, Y. (1998). Survival of *Enterococcus faecalis* in an oligotrophic microcosm: Changes in morphology, development of general stress resistance, and analysis of protein synthesis. *Applied and Environmental Microbiology*, *64*(11), 4238–4245. https://doi.org/10.1128/aem.64.11.4238-4245.1998

Hartman, P. A., Deibel, R. H., & Sieverding, L. M. (2001). Compendium of Methods for The Microbiological Examination of Foods. In *Compendium of Methods for The Microbiological Examination of Foods 4th Edition*. https://doi.org/10.2105/9780875531755

Hasman, H., & Aarestrup, F. M. (2002). *tcrb*, a gene conferring transferable copper resistance in *Enterococcus faecium*: Occurrence, transferability, and linkage to macrolide and glycopeptide resistance. *Antimicrobial Agents and Chemotherapy*. https://doi.org/10.1128/AAC.46.5.1410-1416.2002

Havelaar, A. H., Furuse, K., & Hogeboom, W. M. (1986). Bacteriophages and indicator bacteria in human and animal faeces. *Journal of Applied Bacteriology*. https://doi.org/10.1111/j.1365-2672.1986.tb01081.x

HD. (2021). *Enterococcus*. Hardy Diagnostics. https://catalog.hardydiagnostics.com/cp_prod/Content/hugo/*Enterococcus*.htm

Hegstad, K., Samuelsen, Ø., Janice, J., Elstrøm, P., Kacelnik, O., & Sundsfjord, A. (2020). Forekomst og molekylære genetiske analyser av bakterier med spesielle resistensmønstre i Norge 2019 – rapport fra nasjonalt referanselaboratorium.

Heim, S., Lleo, M. D. M., Bonato, B., Guzman, C. A., & Canepari, P. (2002). The viable but nonculturable state and starvation are different stress responses of *Enterococcus faecalis*, as determined by proteome analysis. *Journal of Bacteriology*, *184*(23), 6739–

6745. https://doi.org/10.1128/JB.184.23.6739-6745.2002

Hidron, A. I., Edwards, J. R., Patel, J., Horan, T. C., Sievert, D. M., Pollock, D. A., & Fridkin, S. K. (2008). Antimicrobial-Resistant Pathogens Associated With Healthcare-Associated Infections: Annual Summary of Data Reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006–2007. *Infection Control & Hospital Epidemiology*. https://doi.org/10.1086/591861

Hollenbeck, B. L., & Rice, L. B. (2012). Intrinsic and acquired resistance mechanisms in *Enterococcus*. In *Virulence*. https://doi.org/10.4161/viru.21282

Homan, W. L., Tribe, D., Poznanski, S., Li, M., Hogg, G., Spalburg, E., Van Embden, J. D. A., & Willems, R. J. L. (2002). Multilocus sequence typing scheme for *Enterococcus faecium*. *Journal of Clinical Microbiology*, *40*(6), 1963–1971. https://doi.org/10.1128/JCM.40.6.1963-1971.2002

International Organization for Standardization. (2019). *ISO 20776-1:2019* Susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices — Part 1: Broth micro-dilution reference method for testing the in vitro activity of antimicrobial agents against rapid. *2019*, 19.

Johnsen, P. J., Østérhus, J. I., Sletvold, H., Sørum, M., Kruse, H., Nielsen, K., Simonsen, G. S., & Sundsfjord, A. (2005). Persistence of animal and human glycopeptide-resistant enterococci on two Norwegian poultry farms formerly exposed to avoparcin is associated with a widespread plasmid-mediated *vanA* element within a polyclonal *Enterococcus faecium* population. *Applied and Environmental Microbiology*, *71*(1), 159–168. https://doi.org/10.1128/AEM.71.1.159-168.2005

Jolley, K. A., & Maiden, M. C. J. (2010). BIGSdb: Scalable analysis of bacterial genome variation at the population level. *BMC Bioinformatics*. https://doi.org/10.1186/1471-2105-11-595

Kirst, H. A., Thompson, D. G., & Nicas, T. I. (1998). Historical yearly usage of vancomycin. *Antimicrobial Agents and Chemotherapy*, *42*(5), 1303–1304. https://doi.org/10.1128/aac.42.5.1303

Klein, G. (2003). Taxonomy, ecology and antibiotic resistance of enterococci from food and the gastro-intestinal tract. *International Journal of Food Microbiology*. https://doi.org/10.1016/S0168-1605(03)00175-2

Koller, M., & Saleh, H. M. (2018). Introductory Chapter: Introducing Heavy Metals. In *Heavy Metals*. https://doi.org/10.5772/intechopen.74783

Koort, J., Coenye, T., Vandamme, P., Sukura, A., & Björkroth, J. (2004). *Enterococcus hermanniensis* sp. nov., from modified-atmosphere-packaged broiler meat and canine tonsils. *International Journal of Systematic and Evolutionary Microbiology*, *54*(5), 1823–1827. https://doi.org/10.1099/ijs.0.63112-0

Kristich, C. J., Rice, L. B., & Arias, C. A. (2014). Enterococcal Infection—Treatment and Antibiotic Resistance. In *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*.

Landecker, H. (2016). Antibiotic Resistance and the Biology of History. *Body and Society*, *22*(4), 19–52. https://doi.org/10.1177/1357034X14561341

Lassen, J., Eckner, K., Hem, L., Nesheim, L., Rimstad, E., & Robertson, L. (2011).

Vurdering av mikrobielle indikatorer for hygieniserte gjødselvarer mv . av organisk opphav.

Law-Brown, J., & Meyers, P. R. (2003). *Enterococcus phoeniculicola* sp. nov., a novel member of the enterococci isolated from the uropygial gland of the Red-billed Woodhoopoe, *Phoeniculus purpureus*. *International Journal of Systematic and Evolutionary Microbiology*, *53*(3), 683–685. https://doi.org/10.1099/ijs.0.02334-0

Layton, B. A., Walters, S. P., Lam, L. H., & Boehm, A. B. (2010). *Enterococcus* species distribution among human and animal hosts using multiplex PCR. *Journal of Applied Microbiology*, *109*(2), 539–547. https://doi.org/10.1111/j.1365-2672.2010.04675.x

Leavis, H. L., Bonten, M. J., & Willems, R. J. (2006). Identification of high-risk enterococcal clonal complexes: global dispersion and antibiotic resistance. In *Current Opinion in Microbiology*. https://doi.org/10.1016/j.mib.2006.07.001

Lebreton, F., Manson, A. L., Saavedra, J. T., Straub, T. J., Earl, A. M., & Gilmore, M. S. (2017). Tracing the Enterococci from Paleozoic Origins to the Hospital. *Cell*, *169*(5), 849-861.e13. https://doi.org/10.1016/j.cell.2017.04.027

Lebreton, F., van Schaik, W., McGuire, A. M., Godfrey, P., Griggs, A., Mazumdar, V., Corander, J., Cheng, L., Saif, S., Young, S., Zeng, Q., Wortman, J., Birren, B., Willems, R. J. L., Earl, A. M., & Gilmore, M. S. (2013). Emergence of epidemic multidrug-resistant *Enterococcus faecium* from animal and commensal strains. *MBio*, *4*(4), 1–10. https://doi.org/10.1128/mBio.00534-13

Lebreton, F., Willems, R. J. L., & Gilmore, M. S. (2014). *Enterococcus* Diversity, Origins in Nature, and Gut Colonization. In *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*.

Leclercq, R., Derlot, E., Duval, J., & Courvalin, P. (1988). Plasmid-Mediated Resistance to Vancomycin and Teicoplanin in *Enterococcus faecium*. In *New England Journal of Medicine* (Vol. 319, Issue 3, pp. 157–161). https://doi.org/10.1056/nejm198807213190307

Lunestad, B. T., Frantzen, S., Svanevik, C. S., Roiha, I. S., & Duinker, A. (2016). Time trends in the prevalence of *Escherichia coli* and enterococci in bivalves harvested in Norway during 2007-2012. *Food Control*, *60*, 289–295. https://doi.org/10.1016/j.foodcont.2015.08.001

Maraccini, P. A., Ferguson, D. M., & Boehm, A. B. (2012). Diurnal variation in *Enterococcus* species composition in polluted ocean water and a potential role for the enterococcal carotenoid in protection against photoinactivation. *Applied and Environmental Microbiology*, *78*(2), 305–310. https://doi.org/10.1128/AEM.06821-11

Marshall, S. H., Donskey, C. J., Hutton-Thomas, R., Salata, R. A., & Rice, L. B. (2002). Gene dosage and linezolid resistance in *Enterococcus faecium* and *Enterococcus faecalis*. *Antimicrobial Agents and Chemotherapy*, *46*(10), 3334–3336. https://doi.org/10.1128/AAC.46.10.3334-3336.2002

Martin, J. D., Mundt, J. O. (1972). Enterococci in Insects. *Applied Microbiology*, *24*(4), 575–580. https://doi.org/10.1128/aem.24.4.575-580.1972

Martinez-Murcia, A. J., & Collins, M. D. (1991). *Enterococcus sulfureus*, a new yellow-pigmented *Enterococcus* species. *FEMS Microbiology Letters*, *80*(1), 69–73.

https://doi.org/10.1111/j.1574-6968.1991.tb04638.x

Martínez, S., López, M., & Bernardo, A. (2003). Thermal inactivation of *Enterococcus faecium*: Effect of growth temperature and physiological state of microbial cells. *Letters in Applied Microbiology*, *37*(6), 475–481. https://doi.org/10.1046/j.1472-765X.2003.01431.x

Masindi, V., & Muedi, K. L. (2018). Environmental Contamination by Heavy Metals. In *Heavy Metals*. https://doi.org/10.5772/intechopen.76082

Matlock, B. (2015). Assessment of Nucleic Acid Purity. *Technical Bulletin NanoDrop Spectrophotometers*, 1–2.

MD. (2007). Om vern av villaksen og ferdigstilling av nasjonale laksevassdrag og laksefjorder St.prp. nr. 32: 1-143. *Det Kongelige Miljøverndepartement*, *32*(32).

Miljøstatus. (2020). *Kommunalt avløpsvann*. https://miljostatus.miljodirektoratet.no/tema/forurensning/kommunalt-avlopsvann/

Moellering, R. C. (1992). Emergence of *Enterococcus* as a Significant Pathogen. *Clinical Infectious Diseases*, *14*(6), 1173–1176. https://doi.org/10.1093/clinids/14.6.1173

Møhlenberg, F., & Riisgård, H. U. (1978). Efficiency of particle retention in 13 species of suspension feeding bivalves. *Ophelia*, *17*(2), 239–246. https://doi.org/10.1080/00785326.1978.10425487

Morandi, S., Cremonesi, P., Povolo, M., & Brasca, M. (2012). *Enterococcus lactis* sp. nov., from Italian raw milk cheeses. *International Journal of Systematic and Evolutionary Microbiology*, *62*(8), 1992–1996. https://doi.org/10.1099/ijs.0.030825-0

Morrison, D., Woodford, N., & Cookson, B. (1997). Enterococci as emerging pathogens of humans. *Journal of Applied Microbiology Symposium Supplement*. https://doi.org/10.1046/j.1365-2672.83.s1.10.x

Mundt, J. O. (1963a). Occurrence of enterococci in animals in a wild environment. *Applied Microbiology*, *11*(October), 136–140. https://doi.org/10.1128/aem.11.2.136-140.1963

Mundt, J. O. (1963b). Occurrence of Enterococci on Plants in a Wild Environment. *Applied Microbiology*, *11*(October), 136–140. https://doi.org/10.1128/aem.11.2.136-140.1963

Mundt, J. Orvin. (1961). Occurrence of Enterococci: Bud, Blossom, and Soil Studies. *Applied Microbiology*, *9*(6), 541–544. https://doi.org/10.1128/aem.9.6.541-544.1961

Mundt, J Orvin, & Graham, W. F. (1986). *Streptococcus faecium* var. *casseliflavus,* nov. var. *95*(6), 2005–2009.

Murray, B. E. (1990). The life and times of the *Enterococcus*. *Clinical Microbiology Reviews*. https://doi.org/10.1128/CMR.3.1.46

Naser, S. M., Vancanneyt, M., De Graef, E., Devriese, L. A., Snauwaert, C., Lefebvre, K., Hoste, B., Švec, P., Decostere, A., Haesebrouck, F., & Swings, J. (2005). *Enterococcus canintestini* sp. nov., from faecal samples of healthy dogs. *International Journal of Systematic and Evolutionary Microbiology*, *55*(5), 2177–2182. https://doi.org/10.1099/ijs.0.63752-0

NLHB. (2021). *L1.1 Om bruk av antimikrobielle midler*. Norsk Legemiddelhåndbok. https://www.legemiddelhandboka.no/L1.1/Om_bruk_av_antimikrobielle_midler

NMKL. (2011). *Enterococcus. Bestemmelse i næringsmidler og fôr (NMKL 68).*

NORM/NORM-VET. (2019). *NORM/NORM-VET 2019. Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway*. *2307*.

Norwegian Food Authority. (2014). *Zinc and Copper in Pig and Poultry Production—Fate and Effects in the Food Chain and the Environment*.

Nowlan, Sandra S., R. H. D. (1967). *Group Q streptococci. I. Ecology, serology, physiology, and relationship to established enterococci*. *94*(2), 291–296.

Oravcova, V., Mihalcin, M., Zakova, J., Pospisilova, L., Masarikova, M., & Literak, I. (2017). Vancomycin-resistant enterococci with *vanA* gene in treated municipal wastewater and their association with human hospital strains. *Science of the Total Environment*. https://doi.org/10.1016/j.scitotenv.2017.07.121

Pal, C., Bengtsson-Palme, J., Rensing, C., Kristiansson, E., & Larsson, D. G. J. (2014). BacMet: Antibacterial biocide and metal resistance genes database. In *Nucleic Acids Research*. https://doi.org/10.1093/nar/gkt1252

Petersen, A., & Jensen, L. B. (2004). Analysis of *gyrA* and *parC* mutations in enterococci from environmental samples with reduced susceptibility to ciprofloxacin. *FEMS Microbiology Letters*, *231*(1), 73–76. https://doi.org/10.1016/S0378-1097(03)00929-7

Poeta, P., Costa, D., Sáenz, Y., Klibi, N., Ruiz-Larrea, F., Rodrigues, J., & Torres, C. (2005). Characterization of antibiotic resistance genes and virulence factors in faecal enterococci of wild animals in Portugal. *Journal of Veterinary Medicine Series B: Infectious Diseases and Veterinary Public Health*, *52*(9), 396–402. https://doi.org/10.1111/j.1439-0450.2005.00881.x

Prieto, A. M. G., Wijngaarden, J., Braat, J. C., Rogers, M. R. C., Majoor, E., Brouwer, E. C., Zhang, X., Bayjanov, J. R., Bonten, M. J. M., Willems, R. J. L., & Van Schaik, W. (2017). The two-component system ChtRS contributes to chlorhexidine tolerance in *Enterococcus faecium*. *Antimicrobial Agents and Chemotherapy*, *61*(5), 1–9. https://doi.org/10.1128/AAC.02122-16

PubMLST. (2020). *Multi-Locus Sequence Typing*.

Rahkila, R., Johansson, P., Säde, E., & Björkroth, J. (2011). Identification of enterococci from broiler products and a broiler processing plant and description of *Enterococcus viikkiensis* sp. nov. *Applied and Environmental Microbiology*, *77*(4), 1196–1203. https://doi.org/10.1128/AEM.02412-10

Ramsey, M., Hartke, A., & Huycke, M. (2014). The Physiology and Metabolism of Enterococci. In *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*.

Ridom GmbH. (2020). *SeqSphere+*. http://www3.ridom.de/seqsphere/

Rodrigues, U., & Collins, M. D. (1990). Phylogenetic analysis of *Streptococcus saccharolyticus* based on 16S rRNA sequencing. *FEMS Microbiology Letters*, *71*(1–2), 231–234. https://doi.org/10.1016/0378-1097(90)90062-U

Sadowy, E. (2018). Linezolid resistance genes and genetic elements enhancing their dissemination in enterococci and streptococci. *Plasmid*, *99*(September), 89–98. https://doi.org/10.1016/j.plasmid.2018.09.011

Sahm, D. F., Kissinger, J., Gilmore, M. S., Murray, P. R., Mulder, R., Solliday, J., & Clarke, B. (1989). In vitro susceptibility studies of vancomycin-resistant *Enterococcus faecalis*. *Antimicrobial Agents and Chemotherapy*. https://doi.org/10.1128/AAC.33.9.1588

Schleifer, K. H., Kilpper-Balz, R. (1984). Transfer of *Streptococcus faecalis* and *Streptococcus faecium* to the genus *Enterococcus* nom. rev. as *Enterococcus faecalis* comb. nov. and *Enterococcus faecium* comb. nov. *International Journal of Systematic Bacteriology*, *34*(1), 31–34. https://doi.org/10.1099/00207713-34-1-31

Schulthess, B., Bloemberg, G. V., Zbinden, R., Böttger, E. C., & Hombach, M. (2014). Evaluation of the bruker MALDI biotyper for identification of gram-positive rods: Development of a diagnostic algorithm for the clinical laboratory. *Journal of Clinical Microbiology*. https://doi.org/10.1128/JCM.02399-13

Sedláček, I., Holochová, P., Mašlaňová, I., Kosina, M., Spröer, C., Bryndová, H., Vandamme, P., Rudolf, I., Hubálek, Z., & Švec, P. (2013). *Enterococcus ureilyticus* sp. nov. and *Enterococcus rotai* sp. nov., two urease-producing enterococci from the environment. *International Journal of Systematic and Evolutionary Microbiology*, *63*(PART2), 502–510. https://doi.org/10.1099/ijs.0.041152-0

Seemann, T. (2020). *ABRicate*. Github. https://github.com/tseemann/abricate

Selden, P. A. (2005). Terrestrialization (Precambrian-Devonian). *ELS*, 1–5. https://doi.org/10.1038/npg.els.0004145

Shaw, T. D., Fairley, D. J., Schneiders, T., Pathiraja, M., Hill, R. L. R., Werner, G., Elborn, J. S., & McMullan, R. (2018). The use of high-throughput sequencing to investigate an outbreak of glycopeptide-resistant *Enterococcus faecium* with a novel quinupristin-dalfopristin resistance mechanism. *European Journal of Clinical Microbiology and Infectious Diseases*, *37*(5), 959–967. https://doi.org/10.1007/s10096-018-3214-x

Sherman, J. M. (1937). THE STREPTOCOCCI. *Bacteriological Reviews*. https://doi.org/10.1128/mmbr.1.1.3-97.1937

Sherman, J. M. (1938). The Enterococci and Related Streptococci. *Journal of Bacteriology*, *35*(2), 81–93. https://doi.org/10.1128/jb.35.2.81-93.1938

Shewmaker, P. L., Steigerwalt, A. G., Nicholson, A. C., Carvalho, M. D. G. S., Facklam, R. R., Whitney, A. M., & Teixeira, L. M. (2011). Reevaluation of the taxonomic status of recently described species of *Enterococcus*: Evidence that *E. thailandicus* is a senior subjective synonym of "*E. sanguinicola*" and confirmation of *E. caccae* as a species distinct from *E. silesiacus*. *Journal of Clinical Microbiology*, *49*(7), 2676–2679. https://doi.org/10.1128/JCM.00399-11

Sistek, V., Maheux, A. F., Boissinot, M., Bernard, K. A., Cantin, P., Cleenwerck, I., de Vos, P., & Bergeron, M. G. (2012). *Enterococcus ureasiticus* sp. nov. and *Enterococcus quebecensis* sp. nov., isolated from water. *International Journal of Systematic and Evolutionary Microbiology*, *62*(6), 1314–1320. https://doi.org/10.1099/ijs.0.029033-0

Statistisk Sentralbyrå. (2020). *Tettsteders befolkning og areal*. https://www.ssb.no/befolkning/folketall/statistikk/tettsteders-befolkning-og-areal

Sukontasing, S., Tanasupawat, S., Moonmangmee, S., Lee, J. S., & Suzuki, K. I. (2007). *Enterococcus camelliae* sp. nov., isolated from fermented tea leaves in Thailand. *International Journal of Systematic and Evolutionary Microbiology*, *57*(9), 2151–2154.

https://doi.org/10.1099/ijs.0.65109-0

Švec, P., Vancanneyt, M., Devriese, L. A., Naser, S. M., Snauwaert, C., Lefebvre, K., Hoste, B., & Swings, J. (2005). *Enterococcus aquimarinus* sp. nov., isolated from sea water. *International Journal of Systematic and Evolutionary Microbiology*, *55*(5), 2183–2187. https://doi.org/10.1099/ijs.0.63722-0

Švec, P., Vancanneyt, M., Koort, J., Naser, S. M., Hoste, B., Vihavainen, E., Vandamme, P., Swings, J., & Bjökroth, J. (2005). *Enterococcus devriesei* sp. nov., associated with animal sources. *International Journal of Systematic and Evolutionary Microbiology*, *55*(6), 2479–2484. https://doi.org/10.1099/ijs.0.63851-0

Švec, P., Vancanneyt, M., Sedláček, I., Naser, S. M., Snauwaert, C., Lefebvre, K., Hoste, B., & Swings, J. (2006). *Enterococcus silesiacus* sp. nov. and *Enterococcus termitis* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, *56*(3), 577–581. https://doi.org/10.1099/ijs.0.63937-0

Tannock, G. W. (1988). The normal microflora: New concepts in health promotion. In *Microbiological Sciences*.

Tendolkar, P. M., Baghdayan, A. S., & Shankar, N. (2003). Pathogenic enterococci: New developments in the 21st century. In *Cellular and Molecular Life Sciences*. https://doi.org/10.1007/s00018-003-3138-0

Treangen, T. J., Ondov, B. D., Koren, S., & Phillippy, A. M. (2014). The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. *Genome Biology*. https://doi.org/10.1186/preaccept-2573980311437212

Tronsmo, A., Gjøen, T., Sørum, H., & Yazdankhah, S. (2016). Antimicrobial resistance due to the use of biocides and heavy metals: a literature review. In *VKM Report 2016: 63*. https://vkm.no/english/riskassessments/allpublications/antimicrobialresistanceduetotheuseofbiocidesandheavymetalsaliteraturereview.4.2375207615dac0245aee25e0.html

Tuma, R. (2003). MALDI-TOF Mass Spectrometry: Getting a Feel for How It Works. *Oncology Times*, *25*(19), 26. https://doi.org/10.1097/01.COT.0000290986.00178.61

Turnidge, J., Kahlmeter, G., & Kronvall, G. (2006). Statistical characterisation of bacterial wild-type MIC value distributions and the determination of epidemiological cut-off values. *Clinical Microbiology and Infection*. https://doi.org/10.1111/j.1469-0691.2006.01377.x

Tyrrell, G. J., Turnbull, L. A., Teixeira, L. M., Lefebvre, J., Carvalho, M. da G. S., Facklam, R. R., & Lovgren, M. (2002). *Enterococcus gilvus* sp. nov. and *Enterococcus pallens* sp. nov. isolated from human clinical specimens. *Journal of Clinical Microbiology*, *40*(4), 1140–1145. https://doi.org/10.1128/JCM.40.4.1140-1145.2002

Ulrich, A., Ott, E., & Müller, M. (2001). *Identification of plant-associated enterococci*. *1999*, 268–278.

University of Leicester. (2020). *Horizontal Gene Transfer*. Virtual Genetics Education Centre. https://www2.le.ac.uk/projects/vgec/schoolsandcolleges/Microbial Sciences/mutation-and-gene-tranfer

Uttley, A. H. C., Collins, C. H., Naidoo, J., & George, R. C. (1988). Vancomycin-Resistant Enterococci. In *The Lancet*. https://doi.org/10.1016/S0140-6736(88)91037-9

Vancanneyt, M., Snauwaert, C., Cleenwerck, I., Baele, M., Descheemaeker, P., Goossens, H., Pot, B., Vandamme, P., Swings, J., Haesebrouck, F., & Devriese, L. A. (2001). *Enterococcus villorum* sp. nov., an enteroadherent bacterium associated with diarrhoea in piglets. *International Journal of Systematic and Evolutionary Microbiology*, *51*(2), 393–400. https://doi.org/10.1099/00207713-51-2-393

Vaux, A., Laguerre, G., Divies, C., & Prevost, H. (1998). *Enterococcus* asini sp. nov. isolated from the caecum of donkeys (*Equus asinus*). *International Journal of Systematic and Evolutionary Microbiology*, *48*(1 998), 383–387.

Vezzulli, L., Grande, C., Reid, P. C., Hélaouët, P., Edwards, M., Höfle, M. G., Brettar, I., Colwell, R. R., & Pruzzo, C. (2016). Climate influence on *Vibrio* and associated human diseases during the past half-century in the coastal North Atlantic. *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.1609157113

Vignaroli, C., Pasquaroli, S., Citterio, B., Di Cesare, A., Mangiaterra, G., Fattorini, D., & Biavasco, F. (2018). Antibiotic and heavy metal resistance in enterococci from coastal marine sediment. *Environmental Pollution*. https://doi.org/10.1016/j.envpol.2018.02.073

Wagenvoort, J. H. T., De Brauwer, E. I. G. B., Penders, R. J. R., van der Linden, C. J., Willems, R. J., Top, J., & Bonten, M. J. (2015). Environmental survival of vancomycin-sensitive ampicillin-resistant *Enterococcus faecium* (AREfm). *European Journal of Clinical Microbiology and Infectious Diseases*, *34*(9), 1901–1903. https://doi.org/10.1007/s10096-015-2430-x

Wastson, Y., Skjerve, E., Yazdankhah, S., Eckner, K., Kapperud, G., Lassen, J. F., Narvhus, J., Nesbakken, T., Robertson, L., Rosnes, J. T., Skjerdal, O. T., & Vold, L. (2019). The Link between Antimicrobial Resistance and the Content of Potentially Toxic Metals in Soil and Fertilising Products. In *European Journal of Nutrition & Food Safety*. https://doi.org/10.9734/ejnfs/2019/v9i430078

Webber, M. A., & Piddock, L. J. V. (2003). The importance of efflux pumps in bacterial antibiotic resistance. *Journal of Antimicrobial Chemotherapy*, *51*(1), 9–11. https://doi.org/10.1093/jac/dkg050

Weideborg, M., Vik, E. A., & Lyngstad, E. (2003). *Riverine inputs and direct discharges to Norwegian coastal waters - 2016.*

Weiner, L. M., Webb, A. K., Limbago, B., Dudeck, M. A., Patel, J., Kallen, A. J., Edwards, J. R., & Sievert, D. M. (2016). Antimicrobial-Resistant Pathogens Associated with Healthcare-Associated Infections: Summary of Data Reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2011-2014. *Infection Control and Hospital Epidemiology*. https://doi.org/10.1017/ice.2016.174

Whitman, R. L., Shively, D. A., Pawlik, H., Nevers, M. B., & Byappanahalli, M. N. (2003). Occurrence of *Escherichia coli* and enterococci in *Cladophora* (Chlorophyta) in nearshore water and beach sand of Lake Michigan. *Applied and Environmental Microbiology*, *69*(8), 4714–4719. https://doi.org/10.1128/AEM.69.8.4714-4719.2003

WHO. (2015). Worldwide country situation analysis: response to antimicrobial resistance. *World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland.*

Williams, A. M., Farrow, J. A. E., & Collins, M. D. (1989). Reverse transcriptase sequencing

of 16S ribosomal RNA from *Streptococcus cecorum*. *Letters in Applied Microbiology*. https://doi.org/10.1111/j.1472-765X.1989.tb00244.x

Wolanin, P. M., Thomason, P. A., & Stock, J. B. (2002). Histidine protein kinases: Key signal transducers outside the animal kingdom. In *Genome Biology*. https://doi.org/10.1186/gb-2002-3-10-reviews3013

Zimbro, M. J., & Power, D. A. (2003). Difco & BBL Manual of Microbiological Culture Media. In *Difco & BBL Manual Manual of Microbiological Culture Media*.

8. Appendix

8.1 MPN/100g for enterococci and *E. coli* samples

LIMS number	From bivalve species	Enterococci MPN/100g	E. coli MPN/100g
2020-672	Pacific oyster	<18	20
2020-320	Ocean quahog	<18	490
2020-990	Great scallop	<18	<18
2020-574	Great scallop	<18	<18
2020-984	Great scallop	<18	20
2020-717	Great scallop	<18	<18
2020-917	Great scallop	<18	20
2020-1012	Great scallop	<18	20
2020-633	Great scallop	<18	<18
2020-803	Great scallop	<18	40
2020-114	European flat oyster	<18	<18
2020-582	European flat oyster	<18	68
2020-322	European flat oyster	<18	130
2020-520	European flat oyster	<18	<18
2020-916	European flat oyster	<18	<18
2020-581	European flat oyster	<18	<18
2020-737	European flat oyster	<18	<18
2020-980	European flat oyster	<18	<18
2020 - 155	European flat oyster	<18	<18
2020-1121	European flat oyster	<18	45
2020-795	European flat oyster	<18	20
2020-319	Carpet shell	<18	40
2020-702	Blue mussel	<18	<18
2020 - 227	Blue mussel	<18	<18
2020 - 273	Blue mussel	<18	20
2020-1093	Blue mussel	<18	78
2020-708	Blue mussel	<18	<18
2020-920	Blue mussel	<18	<18
2019-2189	Blue mussel	<18	20
2020-711	Blue mussel	<18	<18
2020-707	Blue mussel	<18	130
2020-918	Blue mussel	<18	<18
2019-2188	Blue mussel	<18	<18
2020-1094	Blue mussel	<18	<18
2020-706	Blue mussel	<18	<18
2020-919	Blue mussel	<18	<18
2020-709	Blue mussel	<18	20
2020-922	Blue mussel	<18	<18
2020-1103	Blue mussel	<18	230

2020-714	Blue mussel	<18	20
2020-713	Blue mussel	<18	<18
2020-921	Blue mussel	<18	<18
2020-712	Blue mussel	<18	<18
2020-923	Blue mussel	<18	<18
2020-760	Blue mussel	<18	<18
2020-761	Blue mussel	<18	<18
2020-759	Blue mussel	<18	20
2020-762	Blue mussel	<18	<18
2020-749	Blue mussel	<18	<18
2020 - 373	Blue mussel	<18	<18
2020 - 372	Blue mussel	<18	1100
2020 - 278	Blue mussel	<18	1300
2020-697	Blue mussel	<18	45
2020-915	Blue mussel	<18	<18
2020-587	Blue mussel	<18	<18
2020-852	Blue mussel	<18	20
2020 - 153	Blue mussel	<18	20
2020 - 231	Blue mussel	<18	78
2020-1127	Blue mussel	<18	<18
2020-584	Blue mussel	<18	20
2020-750	Blue mussel	<18	<18
2020-987	Blue mussel	<18	<18
2020 - 154	Blue mussel	<18	1100
2020 - 147	Blue mussel	<18	<18
2020 - 151	Blue mussel	<18	78
2020-588	Blue mussel	<18	45
2020 - 146	Blue mussel	<18	<18
2020 - 2	Blue mussel	<18	5400
2020-575	Blue mussel	<18	20
2020-630	Blue mussel	<18	78
2020-851	Blue mussel	<18	45
2020-579	Blue mussel	<18	<18
2020 - 234	Blue mussel	<18	5400
2020-1128	Blue mussel	<18	<18
2020-586	Blue mussel	<18	78
2020-751	Blue mussel	<18	20
2020-986	Blue mussel	<18	1700
2019-2185	Blue mussel	<18	20
2020-1118	Blue mussel	<18	45
2020-583	Blue mussel	<18	45
2020-763	Blue mussel	<18	<18
2020-985	Blue mussel	<18	<18
2020-757	Blue mussel	<18	<18

2020 - 152	Blue mussel	<18	20
2020 - 3	Blue mussel	<18	130
2020-715	Blue mussel	<18	<18
2020-853	Blue mussel	<18	78
2020-1042	Blue mussel	<18	<18
2020-636	Blue mussel	<18	110
2019-2063	Blue mussel	<18	<18
2020-1043	Blue mussel	<18	20
2019-2062	Blue mussel	<18	<18
2020-724	Blue mussel	<18	<18
2020-1044	Blue mussel	<18	<18
2020 - 149	Blue mussel	<18	<18
2020 - 150	Blue mussel	<18	<18
2020 - 4	Blue mussel	<18	130
2020-856	Blue mussel	<18	<18
2019-1950	Blue mussel	<18	<18
2020-721	Blue mussel	<18	<18
2020-753	Blue mussel	<18	20
2020-758	Blue mussel	<18	<18
2020 - 316	Blue mussel	<18	<18
2020-576	Blue mussel	<18	20
2020-747	Blue mussel	<18	<18
2019-2059	Blue mussel	<18	20
2020-719	Blue mussel	<18	<18
2020-754	Blue mussel	<18	45
2020-722	Blue mussel	<18	<18
2020-580	Blue mussel	<18	<18
2020-738	Blue mussel	<18	20
2020-1014	Blue mussel	<18	790
2020-1147	Blue mussel	<18	<18
2020-632	Blue mussel	<18	700
2020-802	Blue mussel	<18	18
2020-631	Blue mussel	<18	68
2020-801	Blue mussel	<18	<18
2020 - 233	Blue mussel	18	<18
2020-748	Blue mussel	18	<18
2020-746	Blue mussel	18	<18
2020-976	Blue mussel	18	78
2020-577	Blue mussel	20	490
2020 - 369	European flat oyster	20	20
2020-699	European flat oyster	20	130
2020 - 270	Cockle	20	20
2020-680	Blue mussel	20	<18
2020-523	Blue mussel	20	<18

2020 - 226	Blue mussel	20	<18
2020-1095	Blue mussel	20	700
2020 - 225	Blue mussel	20	20
2020-522	Blue mussel	20	<18
2020-1100	Blue mussel	20	20
2019-2190	Blue mussel	20	<18
2020 - 228	Blue mussel	20	20
2020-1125	Blue mussel	20	68
2020 - 236	Blue mussel	20	20
2020 - 318	Blue mussel	20	490
2020-978	Blue mussel	20	<18
2020-725	Blue mussel	20	<18
2020 - 376	Blue mussel	20	<18
2019-2057	Blue mussel	20	20
2020-720	Blue mussel	20	68
2020-1104	Blue mussel	20	<18
2020-752	Blue mussel	20	<18
2020 - 315	Blue mussel	20	<18
2020 - 371	Blue mussel	20	78
2020 - 370	Blue mussel	20	<18
2020-1101	Blue mussel	20	20
2020-1096	Blue mussel	20	<18
2020-528	Blue mussel	40	<18
2020-113	Blue mussel	45	<18
2020 - 230	Blue mussel	45	20
2020 - 235	Blue mussel	45	68
2020-519	Blue mussel	45	20
2020-671	Blue mussel	45	45
2020-1119	European flat oyster	45	<18
2020 - 232	Blue mussel	45	40
2020-1117	Blue mussel	45	45
2020-975	Blue mussel	45	<18
2020-1120	Blue mussel	45	45
2020-1146	Blue mussel	45	<18
2020 - 325	Great scallop	45	330
2020-32	Blue mussel	68	<18
2020-756	Blue mussel	68	<18
2020-755	Blue mussel	68	<18
2019-2016	Blue mussel	68	<18
2020 - 374	Blue mussel	78	45
2020-983	Blue mussel	78	<18
2020-1013	Blue mussel	78	<18
2020 - 275	Blue mussel	78	20
2020-1122	European flat oyster	78	<18

2020-744	Great scallop	78	<18
2019-2017	Blue mussel	78	<18
2020-1102	Blue mussel	110	<18
2020-1126	Blue mussel	130	<18
2020-1145	Blue mussel	130	<18
2020 - 314	European flat oyster	130	78
2020 - 375	Blue mussel	130	40
2020 - 274	Blue mussel	140	230
2020-634	Blue mussel	170	20
2020-331	Blue mussel	220	490
2020 - 312	Blue mussel	220	<18
2020-979	Blue mussel	220	<18
2020 - 317	European flat oyster	230	<18
2020-1123	Blue mussel	300	20
2020-1124	Blue mussel	300	<18
2020-1130	Blue mussel	300	<18
2020-1098	Blue mussel	300	78
2020-854	Blue mussel	330	<18
2020-1131	Blue mussel	330	<18
2020 - 311	Great scallop	330	<18
2020-1129	Blue mussel	340	<18
2019-2120	Blue mussel	340	20
2020 - 324	Blue mussel	490	<18
2020-533	Blue mussel	490	<18
2020 - 237	Blue mussel	790	20
2020 - 66	Blue mussel	1300	<18
2019-2186	Blue mussel	1300	<18
2020-855	Blue mussel	2000	<18
2020-989	Blue mussel	3500	<18

8.2 Enterococcal isolates and information

County	Species	From bivalve species	Isolate number	HMR (x)	AMR (x)
Agder	*E. casseliflavus*	Blue mussel	2016-834		*x*
Agder	*E. casseliflavus*	Blue mussel	2020-1098/2		*x*
Agder	*E. casseliflavus*	Blue mussel	2020-1098/4		*x*
Agder	*E. faecalis*	Blue mussel	2016-509		
Agder	*E. faecalis*	European flat oyster	2016-568	x	*x*
Agder	*E. faecalis*	Blue mussel	2016-925/1	x	
Agder	*E. faecalis*	Blue mussel	2016-925/2	x	
Agder	*E. faecalis*	Blue mussel	2020-331/1		*x*
Agder	*E. faecium*	Blue mussel	2016-687		*x*
Agder	*E. faecium*	Blue mussel	2016-873		
Agder	*E. faecium*	Blue mussel	2019 - 2120/1		
Agder	*E. faecium*	Blue mussel	2019 - 2120/2		*x*
Agder	*E. faecium*	Blue mussel	2020-331/2		
Agder	*E. faecium*	Blue mussel	2020-331/3		
Agder	*E. faecium*	Blue mussel	2020-331/4		
Agder	*E. faecium*	Blue mussel	2020-331/5		
Agder	*E. faecium*	Blue mussel	2020-331/6		
Agder	*E. faecium*	Blue mussel	2020-680		
Agder	*E. hirae*	Blue mussel	2016-630		
Agder	*E. hirae*	Blue mussel	2016-835		
Agder	*E. hirae*	Blue mussel	2016-874		
Agder	*E. hirae*	Cockle	2020-270		
Agder	*E. hirae*	Blue mussel	2020-331/7		
Agder	*Enterococcus spp.*	Blue mussel	2020-1098/1		
Agder	*Enterococcus spp.*	Blue mussel	2020-1098/3		
Agder	*Enterococcus spp.*	Blue mussel	2020-1098/5		
Nordland	*E. avium*	Blue mussel	2019 - 2012/1		
Nordland	*E. casseliflavus*	Blue mussel	2020-230/1		
Nordland	*E. casseliflavus*	Blue mussel	2020-275/1		
Nordland	*E. casseliflavus*	Blue mussel	2020-522		
Nordland	*E. casseliflavus*	Blue mussel	2020-524/2		
Nordland	*E. durans*	Blue mussel	2020-1123/2		
Nordland	*E. durans*	Blue mussel	2020-1123/3		
Nordland	*E. durans*	Blue mussel	2020-1124/2		
Nordland	*E. durans*	Blue mussel	2020-226		
Nordland	*E. durans*	Blue mussel	2020-525		
Nordland	*E. durans*	Blue mussel	2020-989/11	x	*x*
Nordland	*E. faecalis*	Blue mussel	2016-1216	x	*x*
Nordland	*E. faecalis*	Blue mussel	2019 - 2014/1	x	*x*
Nordland	*E. faecalis*	Blue mussel	2019 - 2017/1	x	*x*
Nordland	*E. faecalis*	Blue mussel	2020-1095	x	*x*

Nordland	*E. faecalis*	Blue mussel	2020-1124/1	x	
Nordland	*E. faecalis*	Blue mussel	2020-1124/3	x	
Nordland	*E. faecalis*	Blue mussel	2020-1124/4	x	
Nordland	*E. faecalis*	Blue mussel	2020-1124/5	x	
Nordland	*E. faecalis*	Blue mussel	2020-1125	x	
Nordland	*E. faecalis*	Blue mussel	2020-1126/1	x	
Nordland	*E. faecalis*	Blue mussel	2020-1126/3	x	
Nordland	*E. faecalis*	Blue mussel	2020-228	x	
Nordland	*E. faecalis*	Blue mussel	2020-235/2	x	
Nordland	*E. faecalis*	Blue mussel	2020-236	x	
Nordland	*E. faecalis*	Blue mussel	2020-274/2	x	
Nordland	*E. faecalis*	Blue mussel	2020-275/2		*x*
Nordland	*E. faecalis*	Blue mussel	2020-528/1		*x*
Nordland	*E. faecalis*	Blue mussel	2020-528/2		
Nordland	*E. faecalis*	Blue mussel	2020-577/1		
Nordland	*E. faecalis*	Blue mussel	2020-577/2		
Nordland	*E. faecium*	Blue mussel	2016-1098		
Nordland	*E. faecium*	Blue mussel	2016-1129/1		
Nordland	*E. faecium*	Blue mussel	2016-1129/2		
Nordland	*E. faecium*	Blue mussel	2016-1198/1		
Nordland	*E. faecium*	Blue mussel	2016-504		
Nordland	*E. faecium*	Great scallop	2016-538		
Nordland	*E. faecium*	Blue mussel	2016-725		
Nordland	*E. faecium*	Blue mussel	2019 - 2015/1		
Nordland	*E. faecium*	Blue mussel	2019 - 2016/1		
Nordland	*E. faecium*	Blue mussel	2019 - 2117		
Nordland	*E. faecium*	Blue mussel	2019 - 2190		
Nordland	*E. faecium*	Blue mussel	2020-1100		
Nordland	*E. faecium*	Blue mussel	2020-1123/1		
Nordland	*E. faecium*	Blue mussel	2020-1126/2		
Nordland	*E. faecium*	Great scallop	2020-113/2		
Nordland	*E. faecium*	Great scallop	2020-113/3		
Nordland	*E. faecium*	Blue mussel	2020-225		
Nordland	*E. faecium*	Blue mussel	2020-235/1		
Nordland	*E. faecium*	Blue mussel	2020-237/2		
Nordland	*E. faecium*	Blue mussel	2020-237/4		
Nordland	*E. faecium*	Blue mussel	2020-237/5		
Nordland	*E. faecium*	Blue mussel	2020-237/7		
Nordland	*E. faecium*	Blue mussel	2020-274/1		
Nordland	*E. faecium*	Blue mussel	2020-274/3		
Nordland	*E. faecium*	Blue mussel	2020-274/4		
Nordland	*E. faecium*	Blue mussel	2020-274/5		
Nordland	*E. faecium*	Blue mussel	2020-521/1		
Nordland	*E. faecium*	Blue mussel	2020-523		

Nordland	*E. faecium*	Blue mussel	2020-533/1		
Nordland	*E. faecium*	Blue mussel	2020-533/3		
Nordland	*E. faecium*	Blue mussel	2020-533/4		
Nordland	*E. faecium*	Blue mussel	2020-533/5		
Nordland	*E. faecium*	Blue mussel	2020-533/6		
Nordland	*E. faecium*	Blue mussel	2020-533/7		
Nordland	*E. faecium*	Blue mussel	2020-66/2		
Nordland	*E. faecium*	Blue mussel	2020-66/7		
Nordland	*E. faecium*	Blue mussel	2020-66/9		
Nordland	*E. faecium*	Blue mussel	2020-989/1		
Nordland	*E. faecium*	Blue mussel	2020-989/10		
Nordland	*E. faecium*	Blue mussel	2020-989/2		
Nordland	*E. faecium*	Blue mussel	2020-989/3		
Nordland	*E. faecium*	Blue mussel	2020-989/4		
Nordland	*E. faecium*	Blue mussel	2020-989/5		
Nordland	*E. faecium*	Blue mussel	2020-989/6		
Nordland	*E. faecium*	Blue mussel	2020-989/7		
Nordland	*E. faecium*	Blue mussel	2020-989/8		*x*
Nordland	*E. faecium*	Blue mussel	2020-989/9		*x*
Nordland	*E. hirae*	Blue mussel	2016-1198/2		*x*
Nordland	*E. hirae*	Blue mussel	2016-1370		*x*
Nordland	*E. hirae*	Blue mussel	2016-534	x	*x*
Nordland	*E. hirae*	Blue mussel	2020-1123/4	x	*x*
Nordland	*E. hirae*	Great scallop	2020-113/1	x	*x*
Nordland	*E. hirae*	Blue mussel	2020-230/2	x	*x*
Nordland	*E. hirae*	Blue mussel	2020-232/2	x	*x*
Nordland	*E. hirae*	Blue mussel	2020-233	x	*x*
Nordland	*E. hirae*	Blue mussel	2020-237/1	x	
Nordland	*E. hirae*	Blue mussel	2020-237/3	x	
Nordland	*E. hirae*	Blue mussel	2020-237/6	x	
Nordland	*E. hirae*	Blue mussel	2020-237/8	x	
Nordland	*E. hirae*	Blue mussel	2020-275/3	x	
Nordland	*E. hirae*	Blue mussel	2020-521/2	x	
Nordland	*E. hirae*	Blue mussel	2020-66/1	x	
Nordland	*E. hirae*	Blue mussel	2020-66/3	x	
Nordland	*E. hirae*	Blue mussel	2020-66/4	x	
Nordland	*E. hirae*	Blue mussel	2020-66/5	x	
Nordland	*E. hirae*	Blue mussel	2020-66/6	x	
Nordland	*E. hirae*	Blue mussel	2020-66/8		*x*
Nordland	*E. thailandicus*	Blue mussel	2016-1402		*x*
Nordland	*E. thailandicus*	Blue mussel	2020-1126/4		*x*
Nordland	*Enterococcus spp.*	Blue mussel	2020-527		*x*
Rogaland	*E. avium*	Horse mussel	2016-559		*x*
Rogaland	*E. faecalis*	Blue mussel	2016-1148		*x*

Rogaland	*E. faecalis*	Blue mussel	2016-370		*x*
Rogaland	*E. faecalis*	Great scallop	2016-371		*x*
Rogaland	*E. faecalis*	European flat oyster	2016-650		*x*
Rogaland	*E. faecalis*	Blue mussel	2016-813		*x*
Rogaland	*E. faecalis*	European flat oyster	2019-2232		*x*
Rogaland	*E. faecium*	European flat oyster	2016-1065/1		*x*
Rogaland	*E. faecium*	European flat oyster	2016-1065/2		*x*
Rogaland	*E. faecium*	Great scallop	2016-1069/1		*x*
Rogaland	*E. faecium*	Great scallop	2016-1069/2		*x*
Rogaland	*E. faecium*	Great scallop	2016-1241		*x*
Rogaland	*E. faecium*	European flat oyster	2016-1242		*x*
Rogaland	*E. faecium*	Blue mussel	2016-1243/1		*x*
Rogaland	*E. faecium*	European flat oyster	2016-374		*x*
Rogaland	*E. faecium*	Great scallop	2016-651		*x*
Rogaland	*E. faecium*	Great scallop	2020-325/1		*x*
Rogaland	*E. hirae*	Blue mussel	2016-1064/2		*x*
Rogaland	*E. hirae*	Blue mussel	2016-1250		*x*
Rogaland	*E. hirae*	Blue mussel	2016-373		*x*
Rogaland	*E. hirae*	Blue mussel	2016-558		*x*
Rogaland	*E. hirae*	Blue mussel	2016-649		*x*
Rogaland	*E. hirae*	Blue mussel	2016-814		*x*
Rogaland	*E. hirae*	Horse mussel	2016-815		*x*
Rogaland	*E. hirae*	Great scallop	2020-1145/1		*x*
Rogaland	*E. hirae*	Great scallop	2020-1145/2		*x*
Rogaland	*E. hirae*	Great scallop	2020-1145/3		*x*
Rogaland	*E. hirae*	Great scallop	2020-1145/4		*x*
Rogaland	*E. hirae*	Great scallop	2020-744/1		*x*
Rogaland	*E. mundtii*	Blue mussel	2016-1064/1		*x*
Troms & Finnmark	*E. faecium*	Blue mussel	2016-1211/1		*x*
Troms & Finnmark	*E. faecium*	Blue mussel	2016-1211/2		*x*
Troms & Finnmark	*E. faecium*	Blue mussel	2016-1367/1		*x*
Troms & Finnmark	*E. faecium*	Blue mussel	2016-557		*x*
Troms & Finnmark	*E. faecium*	Blue mussel	2020-519/2		*x*
Troms & Finnmark	*E. hirae*	Blue mussel	2016-1127/1		*x*
Troms & Finnmark	*E. hirae*	Blue mussel	2016-1127/2		*x*
Troms & Finnmark	*E. hirae*	Blue mussel	2016-368		*x*
Troms & Finnmark	*E. hirae*	Blue mussel	2016-720		*x*
Troms & Finnmark	*E. hirae*	Blue mussel	2016-954/1		*x*
Troms & Finnmark	*E. hirae*	Blue mussel	2016-954/2		*x*
Troms & Finnmark	*E. hirae*	Blue mussel	2020-519/1		*x*
Trøndelag	*E. avium*	Blue mussel	2020-1117/1		*x*
Trøndelag	*E. avium*	Blue mussel	2020-1117/2		*x*
Trøndelag	*E. durans*	Blue mussel	2020-1130/1		*x*
Trøndelag	*E. durans*	Blue mussel	2020-1130/2		*x*

Trøndelag	*E. durans*	Blue mussel	2020-1130/3		*x*
Trøndelag	*E. durans*	Blue mussel	2020-1130/4		*x*
Trøndelag	*E. durans*	Blue mussel	2020-1130/5		*x*
Trøndelag	*E. durans*	Blue mussel	2020-1130/6		*x*
Trøndelag	*E. durans*	Blue mussel	2020-1131/1		*x*
Trøndelag	*E. durans*	Blue mussel	2020-1131/2		*x*
Trøndelag	*E. durans*	Blue mussel	2020-1131/3		*x*
Trøndelag	*E. durans*	Blue mussel	2020-1131/4		*x*
Trøndelag	*E. durans*	Blue mussel	2020-854/1		
Trøndelag	*E. durans*	Blue mussel	2020-854/2		
Trøndelag	*E. durans*	Blue mussel	2020-854/3		
Trøndelag	*E. durans*	Blue mussel	2020-854/4		
Trøndelag	*E. durans*	Blue mussel	2020-854/5		
Trøndelag	*E. durans*	Blue mussel	2020-855/10		
Trøndelag	*E. durans*	Blue mussel	2020-855/11		
Trøndelag	*E. durans*	Blue mussel	2020-855/12		
Trøndelag	*E. durans*	Blue mussel	2020-855/4		
Trøndelag	*E. faecalis*	Blue mussel	2016-1030/1		
Trøndelag	*E. faecalis*	Blue mussel	2016-1030/2		
Trøndelag	*E. faecalis*	Blue mussel	2016-1037/1		
Trøndelag	*E. faecalis*	Blue mussel	2016-1037/2		
Trøndelag	*E. faecalis*	Blue mussel	2016-1173/2		
Trøndelag	*E. faecalis*	Blue mussel	2016-1179/1		
Trøndelag	*E. faecalis*	Blue mussel	2016-1179/2		
Trøndelag	*E. faecalis*	Blue mussel	2016-1199/2		
Trøndelag	*E. faecalis*	Great scallop	2016-1371		
Trøndelag	*E. faecalis*	Blue mussel	2016-259		
Trøndelag	*E. faecalis*	Blue mussel	2016-321/1		
Trøndelag	*E. faecalis*	Blue mussel	2016-445/1		
Trøndelag	*E. faecalis*	Blue mussel	2016-502		
Trøndelag	*E. faecalis*	Blue mussel	2016-730		
Trøndelag	*E. faecalis*	Blue mussel	2016-828/1		
Trøndelag	*E. faecalis*	Blue mussel	2016-828/2		
Trøndelag	*E. faecalis*	Blue mussel	2016-832		
Trøndelag	*E. faecalis*	Blue mussel	2016-945/1		
Trøndelag	*E. faecalis*	Blue mussel	2020-1129/1		
Trøndelag	*E. faecalis*	Blue mussel	2020-32/2		
Trøndelag	*E. faecalis*	Blue mussel	2020-671/1		
Trøndelag	*E. faecium*	Blue mussel	2016-1031/1		
Trøndelag	*E. faecium*	Blue mussel	2016-1031/2		
Trøndelag	*E. faecium*	Blue mussel	2016-1032		
Trøndelag	*E. faecium*	Blue mussel	2016-1033/1		
Trøndelag	*E. faecium*	Blue mussel	2016-1033/2		
Trøndelag	*E. faecium*	Blue mussel	2016-1034/1		

Trøndelag	*E. faecium*	Blue mussel	2016-1034/2		
Trøndelag	*E. faecium*	Blue mussel	2016-1035/1		
Trøndelag	*E. faecium*	Blue mussel	2016-1035/2		
Trøndelag	*E. faecium*	Blue mussel	2016-1036		
Trøndelag	*E. faecium*	Blue mussel	2016-1039/1		
Trøndelag	*E. faecium*	Blue mussel	2016-1041		
Trøndelag	*E. faecium*	Blue mussel	2016-1042/1		
Trøndelag	*E. faecium*	Blue mussel	2016-1042/2		
Trøndelag	*E. faecium*	Blue mussel	2016-1072/1		
Trøndelag	*E. faecium*	Blue mussel	2016-1072/2		
Trøndelag	*E. faecium*	Great scallop	2016-1073		
Trøndelag	*E. faecium*	Blue mussel	2016-1076/1		
Trøndelag	*E. faecium*	Blue mussel	2016-1076/2		
Trøndelag	*E. faecium*	Blue mussel	2016-1131/1		
Trøndelag	*E. faecium*	Blue mussel	2016-1131/2		
Trøndelag	*E. faecium*	Blue mussel	2016-1132		
Trøndelag	*E. faecium*	Blue mussel	2016-1137		
Trøndelag	*E. faecium*	Blue mussel	2016-1139		
Trøndelag	*E. faecium*	Blue mussel	2016-1145/1		
Trøndelag	*E. faecium*	Blue mussel	2016-1145/2		
Trøndelag	*E. faecium*	Blue mussel	2016-1168/1		
Trøndelag	*E. faecium*	Blue mussel	2016-1168/2		
Trøndelag	*E. faecium*	Blue mussel	2016-1172/1		
Trøndelag	*E. faecium*	Blue mussel	2016-1177/1		
Trøndelag	*E. faecium*	Blue mussel	2016-1199/1		
Trøndelag	*E. faecium*	Blue mussel	2016-1200		
Trøndelag	*E. faecium*	Blue mussel	2016-1201/1		
Trøndelag	*E. faecium*	Blue mussel	2016-1201/2		
Trøndelag	*E. faecium*	Blue mussel	2016-1214/1		
Trøndelag	*E. faecium*	Blue mussel	2016-1214/2		
Trøndelag	*E. faecium*	Blue mussel	2016-1238		
Trøndelag	*E. faecium*	Blue mussel	2016-1249		
Trøndelag	*E. faecium*	Blue mussel	2016-1255		
Trøndelag	*E. faecium*	Blue mussel	2016-1290		
Trøndelag	*E. faecium*	Blue mussel	2016-1292		
Trøndelag	*E. faecium*	Blue mussel	2016-1295		
Trøndelag	*E. faecium*	Blue mussel	2016-1296		
Trøndelag	*E. faecium*	Blue mussel	2016-1331		
Trøndelag	*E. faecium*	Blue mussel	2016-1372		
Trøndelag	*E. faecium*	Blue mussel	2016-1374		
Trøndelag	*E. faecium*	Blue mussel	2016-1375		
Trøndelag	*E. faecium*	Blue mussel	2016-1393		
Trøndelag	*E. faecium*	Blue mussel	2016-1399		
Trøndelag	*E. faecium*	Blue mussel	2016-260		

Trøndelag	*E. faecium*	Blue mussel	2016-261		
Trøndelag	*E. faecium*	Blue mussel	2016-262		
Trøndelag	*E. faecium*	Blue mussel	2016-264		
Trøndelag	*E. faecium*	Great scallop	2016-320/1		
Trøndelag	*E. faecium*	Blue mussel	2016-375		
Trøndelag	*E. faecium*	Blue mussel	2016-376		
Trøndelag	*E. faecium*	Blue mussel	2016-377		
Trøndelag	*E. faecium*	Blue mussel	2016-380		
Trøndelag	*E. faecium*	Great scallop	2016-439/1		
Trøndelag	*E. faecium*	Blue mussel	2016-447/1		
Trøndelag	*E. faecium*	Blue mussel	2016-498		
Trøndelag	*E. faecium*	Blue mussel	2016-499		
Trøndelag	*E. faecium*	Blue mussel	2016-501		
Trøndelag	*E. faecium*	Blue mussel	2016-503		
Trøndelag	*E. faecium*	Blue mussel	2016-506		
Trøndelag	*E. faecium*	European flat oyster	2016-507		
Trøndelag	*E. faecium*	Horse mussel	2016-508		
Trøndelag	*E. faecium*	Blue mussel	2016-536		
Trøndelag	*E. faecium*	Blue mussel	2016-537		
Trøndelag	*E. faecium*	Blue mussel	2016-563		
Trøndelag	*E. faecium*	Blue mussel	2016-565		
Trøndelag	*E. faecium*	Blue mussel	2016-566		
Trøndelag	*E. faecium*	Blue mussel	2016-636		
Trøndelag	*E. faecium*	Blue mussel	2016-644		
Trøndelag	*E. faecium*	Blue mussel	2016-678		
Trøndelag	*E. faecium*	Blue mussel	2016-682		
Trøndelag	*E. faecium*	Blue mussel	2016-685		
Trøndelag	*E. faecium*	Blue mussel	2016-686		
Trøndelag	*E. faecium*	Blue mussel	2016-727		
Trøndelag	*E. faecium*	Blue mussel	2016-729		
Trøndelag	*E. faecium*	Blue mussel	2016-731		
Trøndelag	*E. faecium*	Blue mussel	2016-827		
Trøndelag	*E. faecium*	Blue mussel	2016-833		
Trøndelag	*E. faecium*	Blue mussel	2016-878		
Trøndelag	*E. faecium*	Blue mussel	2016-883/1		
Trøndelag	*E. faecium*	Blue mussel	2016-883/2		
Trøndelag	*E. faecium*	Blue mussel	2016-930/1		
Trøndelag	*E. faecium*	Blue mussel	2016-930/2		
Trøndelag	*E. faecium*	Blue mussel	2019 - 2057/1		
Trøndelag	*E. faecium*	Blue mussel	2019 - 2186/1		
Trøndelag	*E. faecium*	Blue mussel	2019 - 2186/2		
Trøndelag	*E. faecium*	Blue mussel	2020-1104		
Trøndelag	*E. faecium*	Blue mussel	2020-1129/3		
Trøndelag	*E. faecium*	Blue mussel	2020-1129/5		

Trøndelag	*E. faecium*	Blue mussel	2020-1129/6		
Trøndelag	*E. faecium*	Blue mussel	2020-1129/8		
Trøndelag	*E. faecium*	Blue mussel	2020-312/1		
Trøndelag	*E. faecium*	Blue mussel	2020-312/3		
Trøndelag	*E. faecium*	Blue mussel	2020-312/4		
Trøndelag	*E. faecium*	Blue mussel	2020-32/1		
Trøndelag	*E. faecium*	Blue mussel	2020-32/3		
Trøndelag	*E. faecium*	Blue mussel	2020-324/1		
Trøndelag	*E. faecium*	Blue mussel	2020-324/2		
Trøndelag	*E. faecium*	Blue mussel	2020-324/3		
Trøndelag	*E. faecium*	Blue mussel	2020-324/4		
Trøndelag	*E. faecium*	Blue mussel	2020-324/5		
Trøndelag	*E. faecium*	Blue mussel	2020-324/6		
Trøndelag	*E. faecium*	Blue mussel	2020-324/7		
Trøndelag	*E. faecium*	Blue mussel	2020-332		
Trøndelag	*E. faecium*	Blue mussel	2020-368		
Trøndelag	*E. faecium*	Blue mussel	2020-374/1		
Trøndelag	*E. faecium*	Blue mussel	2020-374/3		
Trøndelag	*E. faecium*	Blue mussel	2020-375/1		
Trøndelag	*E. faecium*	Blue mussel	2020-375/2		
Trøndelag	*E. faecium*	Blue mussel	2020-375/3		
Trøndelag	*E. faecium*	Blue mussel	2020-375/4		
Trøndelag	*E. faecium*	Blue mussel	2020-634/2		
Trøndelag	*E. faecium*	Blue mussel	2020-635/1		
Trøndelag	*E. faecium*	Blue mussel	2020-635/2		
Trøndelag	*E. faecium*	Blue mussel	2020-635/3		
Trøndelag	*E. faecium*	Blue mussel	2020-671/2		
Trøndelag	*E. faecium*	Blue mussel	2020-720		
Trøndelag	*E. faecium*	Blue mussel	2020-746		
Trøndelag	*E. faecium*	Blue mussel	2020-755/2		
Trøndelag	*E. faecium*	Blue mussel	2020-755/3		
Trøndelag	*E. faecium*	Blue mussel	2020-756/3		
Trøndelag	*E. faecium*	Blue mussel	2020-855/1		
Trøndelag	*E. faecium*	Blue mussel	2020-855/13		
Trøndelag	*E. faecium*	Blue mussel	2020-855/2		
Trøndelag	*E. faecium*	Blue mussel	2020-855/3		
Trøndelag	*E. faecium*	Blue mussel	2020-855/5		
Trøndelag	*E. faecium*	Blue mussel	2020-855/6		
Trøndelag	*E. faecium*	Blue mussel	2020-855/7		
Trøndelag	*E. faecium*	Blue mussel	2020-855/8		
Trøndelag	*E. faecium*	Blue mussel	2020-855/9		
Trøndelag	*E. faecium*	Blue mussel	2020-975/1		
Trøndelag	*E. faecium*	Blue mussel	2020-975/2		
Trøndelag	*E. faecium*	Blue mussel	2020-978		

Trøndelag	*E. faecium*	Blue mussel	2020-979/1		*x*
Trøndelag	*E. faecium*	Blue mussel	2020-979/2		*x*
Trøndelag	*E. faecium*	Blue mussel	2020-979/3		*x*
Trøndelag	*E. faecium*	Blue mussel	2020-979/4	x	
Trøndelag	*E. faecium*	Blue mussel	2020-979/5	x	
Trøndelag	*E. gallinarum*	Blue mussel	2016-1245	x	
Trøndelag	*E. hirae*	Blue mussel	2016-1075/1	x	
Trøndelag	*E. hirae*	Blue mussel	2016-1075/2	x	
Trøndelag	*E. hirae*	Blue mussel	2016-1169/1	x	
Trøndelag	*E. hirae*	Blue mussel	2016-1169/2	x	
Trøndelag	*E. hirae*	Blue mussel	2016-1170		*x*
Trøndelag	*E. hirae*	Blue mussel	2016-1171/1		
Trøndelag	*E. hirae*	Blue mussel	2016-1171/2		
Trøndelag	*E. hirae*	Blue mussel	2016-1172/2		
Trøndelag	*E. hirae*	Blue mussel	2016-1244/1		
Trøndelag	*E. hirae*	Blue mussel	2016-1248		
Trøndelag	*E. hirae*	Blue mussel	2016-1252		
Trøndelag	*E. hirae*	Blue mussel	2016-1256		
Trøndelag	*E. hirae*	Blue mussel	2016-1291		
Trøndelag	*E. hirae*	Blue mussel	2016-1378		
Trøndelag	*E. hirae*	Blue mussel	2016-1396		
Trøndelag	*E. hirae*	Blue mussel	2016-1398		
Trøndelag	*E. hirae*	Blue mussel	2016-1400		
Trøndelag	*E. hirae*	Blue mussel	2016-366		
Trøndelag	*E. hirae*	Blue mussel	2016-379		
Trøndelag	*E. hirae*	Blue mussel	2016-500		
Trøndelag	*E. hirae*	Blue mussel	2016-505		
Trøndelag	*E. hirae*	Blue mussel	2016-564/1		
Trøndelag	*E. hirae*	Blue mussel	2016-637		
Trøndelag	*E. hirae*	Blue mussel	2016-639		
Trøndelag	*E. hirae*	Blue mussel	2016-683		
Trøndelag	*E. hirae*	Blue mussel	2016-829		
Trøndelag	*E. hirae*	Blue mussel	2016-830		
Trøndelag	*E. hirae*	Blue mussel	2016-831		
Trøndelag	*E. hirae*	Blue mussel	2016-836		
Trøndelag	*E. hirae*	Blue mussel	2016-882/1		
Trøndelag	*E. hirae*	Blue mussel	2016-882/2		
Trøndelag	*E. hirae*	Blue mussel	2016-942/1		
Trøndelag	*E. hirae*	Blue mussel	2016-942/2		
Trøndelag	*E. hirae*	Blue mussel	2016-944		
Trøndelag	*E. hirae*	Blue mussel	2016-945/2		
Trøndelag	*E. hirae*	Blue mussel	2020-1129/2		
Trøndelag	*E. hirae*	Blue mussel	2020-1129/4		
Trøndelag	*E. hirae*	Blue mussel	2020-312/2		

Trøndelag	*E. hirae*	Blue mussel	2020-312/5		
Trøndelag	*E. hirae*	Blue mussel	2020-312/6		
Trøndelag	*E. hirae*	Blue mussel	2020-318		
Trøndelag	*E. hirae*	Blue mussel	2020-374/2		
Trøndelag	*E. hirae*	Blue mussel	2020-376		
Trøndelag	*E. hirae*	Blue mussel	2020-634/1		
Trøndelag	*E. hirae*	Blue mussel	2020-634/3		
Trøndelag	*E. hirae*	Blue mussel	2020-634/4		
Trøndelag	*E. hirae*	Blue mussel	2020-634/5		
Trøndelag	*E. hirae*	Blue mussel	2020-635/4		
Trøndelag	*E. hirae*	Blue mussel	2020-635/5		
Trøndelag	*E. hirae*	Blue mussel	2020-725		
Trøndelag	*E. hirae*	Blue mussel	2020-748		
Trøndelag	*E. hirae*	Blue mussel	2020-755/1		
Trøndelag	*E. hirae*	Blue mussel	2020-756/1		
Trøndelag	*E. hirae*	Blue mussel	2020-756/2		
Trøndelag	*E. mundtii*	Blue mussel	2020-1129/7		
Trøndelag	*E. villorum*	Blue mussel	2020-752		
Vestland	*E. faecalis*	European flat oyster	2016-1215		
Vestland	*E. faecalis*	European flat oyster	2016-1251		
Vestland	*E. faecalis*	Blue mussel	2016-631		
Vestland	*E. faecalis*	Blue mussel	2019-2231		
Vestland	*E. faecalis*	European flat oyster	2020-1122/2		
Vestland	*E. faecalis*	European flat oyster	2020-1122/3		
Vestland	*E. faecalis*	Blue mussel	2020-1146/1		
Vestland	*E. faecalis*	Blue mussel	2020-1146/2		
Vestland	*E. faecalis*	European flat oyster	2020-314/1		
Vestland	*E. faecalis*	European flat oyster	2020-314/2		
Vestland	*E. faecalis*	European flat oyster	2020-314/3		
Vestland	*E. faecalis*	European flat oyster	2020-314/4		
Vestland	*E. faecalis*	Blue mussel	2020-983/2		
Vestland	*E. faecalis*	Blue mussel	2020-983/3		
Vestland	*E. faecium*	European flat oyster	2016-1063/1		
Vestland	*E. faecium*	European flat oyster	2016-1167/1		
Vestland	*E. faecium*	European flat oyster	2016-1167/2		
Vestland	*E. faecium*	European flat oyster	2016-1176		
Vestland	*E. faecium*	European flat oyster	2016-1297		
Vestland	*E. faecium*	European flat oyster	2016-199		
Vestland	*E. faecium*	Blue mussel	2016-204		
Vestland	*E. faecium*	European flat oyster	2016-265		
Vestland	*E. faecium*	European flat oyster	2016-319/1		
Vestland	*E. faecium*	Blue mussel	2016-441/1		
Vestland	*E. faecium*	European flat oyster	2016-443/1		
Vestland	*E. faecium*	Blue mussel	2016-444/1		

Vestland	*E. faecium*	European flat oyster	2016-535		
Vestland	*E. faecium*	Great scallop	2016-641		
Vestland	*E. faecium*	Banded carpet shell	2016-643		
Vestland	*E. faecium*	Blue mussel	2019-1762		
Vestland	*E. faecium*	Blue mussel	2019-1764		
Vestland	*E. faecium*	Blue mussel	2019-1814		
Vestland	*E. faecium*	Blue mussel	2019-1911		
Vestland	*E. faecium*	European flat oyster	2019-2233		
Vestland	*E. faecium*	Blue mussel	2019-2238		
Vestland	*E. faecium*	European flat oyster	2020-1119/1		
Vestland	*E. faecium*	European flat oyster	2020-1119/2		
Vestland	*E. faecium*	Great scallop	2020-311/1		
Vestland	*E. faecium*	Great scallop	2020-311/3		
Vestland	*E. faecium*	Great scallop	2020-311/4		
Vestland	*E. faecium*	European flat oyster	2020-317/1		
Vestland	*E. faecium*	European flat oyster	2020-317/2		
Vestland	*E. faecium*	European flat oyster	2020-317/3		
Vestland	*E. faecium*	European flat oyster	2020-317/5		
Vestland	*E. faecium*	Blue mussel	2020-370		
Vestland	*E. faecium*	European flat oyster	2020-699		
Vestland	*E. gallinarum*	Blue mussel	2020-1120/1		
Vestland	*E. gallinarum*	Blue mussel	2020-1120/2		
Vestland	*E. hirae*	European flat oyster	2016-1063/2		
Vestland	*E. hirae*	Blue mussel	2016-1146/1		
Vestland	*E. hirae*	Blue mussel	2016-1146/2		
Vestland	*E. hirae*	Blue mussel	2016-1147/1		
Vestland	*E. hirae*	Blue mussel	2016-1147/2		
Vestland	*E. hirae*	Blue mussel	2016-1240		
Vestland	*E. hirae*	Blue mussel	2016-1344		
Vestland	*E. hirae*	Blue mussel	2016-1395		
Vestland	*E. hirae*	Blue mussel	2016-201		
Vestland	*E. hirae*	Blue mussel	2016-202		
Vestland	*E. hirae*	Blue mussel	2016-810		
Vestland	*E. hirae*	European flat oyster	2016-921		*x*
Vestland	*E. hirae*	European flat oyster	2016-923		
Vestland	*E. hirae*	Blue mussel	2020-315		
Vestland	*E. hirae*	European flat oyster	2020-317/4		
Vestland	*E. hirae*	European flat oyster	2020-369		
Vestland	*E. hirae*	Blue mussel	2020-371		*x*
Vestland	*E. mundtii*	Blue mussel	2020-983/1		*x*
Vestland	*E. thailandicus*	Blue mussel	2020-1013/1		
Vestland	*E. thailandicus*	Blue mussel	2020-1013/3		
Vestland	*Enterococcus spp.*	Blue mussel	2020-1013/2		

8.3 Recipes for growth media and other solutions

KF Streptococcus Broth, single

For MPN procedure, yields 1L

- 56,4 g KF Streptococcus Broth powder
- 1 L dH_2O
- Scale, Mettler Toledo PG5002-S DeltaRange®
- Erlenmeyer flask of 1L
- Magnetic stirrer, IKA® RCT basic IKAMAG™ Safety Control
- Magnet
- Tin foil
- Water bath, 100 °C
- Volumetric flask weight ring
- Peristaltic pump (INTEGRA Biosciences AG, Watson & Marlow, Model DOSE IT P910)
- Sterilizable 10 mL glass tubes
- Autoclave, 121 ± 2°C
- pH meter, inoLab® pH 7110, WTW

The powder was weighed in an erlenmeyer flask, later the dH_2O was added and the solution was well mixed on a magnetic stirrer. At this point the solution was dark purple. The solution was then brought to a boil in a water bath at 100 °C. The erlenmeyer flask was covered with tin foil and stabilized in the water with a volumetric flask weight ring around its bottleneck. The solution was boiled for about 15 minutes, ensuring that the powder was completely dissolved. When the solution had cooled properly, the pH was measured, it should be 7.2 ± 0.2. The solution was then allocated into 10 ml glass tubes using a peristaltic pump, setting the volume at 10 ml. After plastic lids were put on top of each tube, they were autoclaved at 121°Cfor 10 minutes. At this point, the color of the solution changed from deep purple to red. The tubes were stored in a cool room (+2-4 °C).

KF Streptococcus Broth, double

For MPN procedure, yields 1L

- 112,8 g KF Streptococcus Broth powder
- 1 L dH_2O
- Scale, Mettler Toledo PG5002-S DeltaRange®
- Erlenmeyer flask of 1L
- Magnetic stirrer, IKA® RCT basic IKAMAG™ Safety Control
- Magnet
- Tin foil
- Water bath, 100 °C
- Volumetric flask weight ring
- Peristaltic pump (INTEGRA Biosciences AG, Watson & Marlow, Model DOSE IT P910)
- Sterilizable 20 mL glass tubes
- Autoclave, 121 ± 2 °C
- pH meter, inoLab® pH 7110, WTW

Same procedure as described above for KF Streptococcus Broth, single. The powder was weighed in an erlenmeyer flask, later the dH_2O was added and the solution was well mixed on a magnetic stirrer. At this point the solution was dark purple. The solution was then brought to a boil in a water bath at 100 °C. The erlenmeyer flask was covered with tin foil and stabilized in the water with a volumetric flask weight ring around its bottleneck. The solution was boiled for about 15 minutes, ensuring that the powder was completely dissolved. When the solution had cooled properly, the pH was measured, it should be 7.2 ± 0.2. The solution was then allocated into 20 ml glass tubes using a peristaltic pump, setting the volume at 10 ml. After plastic lids were put on top of each tube, they were autoclaved at 121°Cfor 10 minutes. At this point, the color of the solution changed from deep purple to red. The tubes were stored in a cool room (+2-4 °C).

<u>m-Enterococcus Agar (ENT)</u>
Yields 1L, enough for ca. 60 Petri dishes

- 42g of m-Enterococcus Agar powder
- 1 L dH_2O

- Scale, Mettler Toledo PG5002-S DeltaRange®
- Erlenmeyer flask of 1L
- Magnetic stirrer, IKA® RCT basic IKAMAG™ Safety Control
- Magnet
- Tin foil
- Water bath, 100 °C
- Volumetric flask weight ring
- Water bath, 45°C
- pH meter, inoLab® pH 7110, WTW
- Sterile Petri dishes, 9 cm

The powder was weighed in an erlenmeyer flask, later the dH_2O was added and the solution was well mixed on a magnetic stirrer. At this point the color was light yellow. The flask was sealed with a tin foil and a stabilizing iron ring put around it. Then the flask was put in a water bath at 100 °C and boiled for ca. 15 minutes. It could be tricky find the optimal boiling time for this medium, as the solution very sudden shifts in color and then loses its properties. Frequent observation of this medium while boiling was necessary. For smaller volumes, boiling time was shorter. The solution was ready when it turned peach-colored, pink or a very deep yellow. It was over-cooked if it turned red and should be disposed. As soon as the solution shifts in color, the flask should be placed in a waterbath of 45°C to cool down. pH was then measured and should be 7.2 ± 0.2. After the solution had cooled down to 45°C, the Petri dishes were made and stored in a cool room (+2-4 °C).

<u>Vancomycin stock solution for agar plates, 1 mg/ml</u>
Yields 15 mL.
Dilution 1:100

- 16,6 mg Vancomycin hydrochloride from Streptomyces Orientalis, powder
- 15 mL dH_2O
- Scale, Mettler Toledo PG2005-S

The Vancomycin powder was weighed in a fume cupboard into a small cylindrical container which had a lid to it. In a 50 mL beaker, around 20 mL of dH_2O was added. From this beaker, 7.5 mL of dH_2O was added twice to the container with the Vancomycin powder, yielding a

total volume of 15 mL. The solution was shaken with the lid on for 10 seconds to properly dissolve the powder. The Vancomycin solution was then sterile filtered and directly allocated in eight aliquots of 1.5 mL into eppendorf tubes, and then put in a freezer (-25°C) for later use. It can remain frozen for 1 year before expiring.

Enterococcus agar plates containing Vancomycin
Yields 100 ml of agar, yields 5-6 agar plates.

- 4,2 g m-Enterococcus Agar powder
- 100 mL dH_2O
- 0.6 ml Vancomycin 1 mg/ml stock solution
- Scale, Mettler Toledo PG5002-S DeltaRange®

Per 100 ml of agar, 0.6 mL of the Vancomycin stock solution is added. These agar plates expire after a couple of days; therefore, they should be made for using the same day. The Enterococcus agar solution was prepared by following the same procedure as when making them without Vancomycin. After the Enterococcus solution changed color to pink, it was immediately transferred to a water bath, cooling the solution down to 45 °C. After 1 hour, the erlenmeyer flask containing the Enterococcus agar, was removed from the water bath and 0.6 mL of the Vancomycin stock solution was added by pipette. The flask was stirred gently, and the solution allocated in Petri dishes, drying for 15 minutes before storing in the cool room with lids on, lying upside-down.

75% Glycerol solution
Yields 100 ml

- 75 mL Glycerol
- 25 mL dH_2O
- Graduated cylinder, 100 mL
- Autoclave, 121 ± 2 °C

75 mL Glycerol was measured in a graduated cylinder. 25 mL of dH_2O was added to the 75 mL of Glycerol, and then poured together into a small bottle with a screw cap. The solution

was autoclaved at 121℃ for 10 mins. After autoclaving, the solution was allocated into four tubes with screw cap à 25 mL and stored in room temperature.

Nutrient Broth

Yields 100 mL

- 1,3 g Nutrient Broth powder
- 100 mL dH_2O
- Scale, Mettler Toledo PG5002-S DeltaRange®
- Erlenmeyer flask of 100 mL
- Autoclave, 121 ± 2 ℃
- Sterile Eppendorf tubes

The powder was weighed and mixed with 100 mL dH_2O in a 100 mL Erlenmeyer flask. A tin foil lid was put on the flask and the solution was autoclaved for 15 minutes at 121 C. After autoclaving, the solution was allocated into aliquots of 500 µl in Eppendorf tubes and stored in a cool room (+2-4 C).

Bile Esculinazid Agar (ESC)

Yields 100 mL

- 5,7 g Bile Esculinazid Agar powder
- 100 mL dH_2O
- Erlenmeyer flask of 100 mL
- Sterile Petri dishes, 9 cm
- Scale, Mettler Toledo PG5002-S DeltaRange®
- Water bath, 100 ℃
- Water bath, 45.0 ± 1.0 ℃
- Glass flask, sterilizable
- Autoclave, 121 ± 2 ℃
- pH meter, inoLab® pH 7110, WTW

5,7 grams of ESC powder was weighed in an Erlenmeyer flask. 100 mL dH_2O was then added, and the solution brought to a boil. pH should be 7.1 ± 0.2 °C. Autoclaved for 15 minutes at 121 °C. Before use, the solution was tempered in a water bath at 45 °C before being poured into petri dishes.

Gram staining

- Potassium Hydroxide (KOH)
- Object glass
- Disposable inoculation loops, small loop size

With an inoculation loop, a colony of the bacteria was transferred to the object glass. A drop of KOH was applied on top, and a new inoculation loop was used to stir and look for whether there would be a thread (Gram negative) or none (Gram positive).

Catalase test

- Hydrogen peroxide (H_2O_2)
- Object glass
- Disposable inoculation loops
- Fume cupboard

With an inoculation loop, a colony of the bacteria was transferred to the object glass. A drop of H2O2 was applied on top. If the bacteria are Catalase negative, there will be no foaming. If it is Catalase positive, small carbonic bubbles will appear and sometimes even splash, hence why this test is done in a fume cupboard.

Shellfish processing method, extraction of Enterococcus from raw sample, MPN

Every Tuesday, a varying number of samples containing Blue mussel (*Mytilus edulis)* is brought to the Institute of Marine Research (IMR) in Bergen for a standard *E. coli* enumeration analysis. This analysis is performed weekly by the staff working at the microbiology lab at IMR. I am so fortunate to be allowed to use these same samples for my master research after the staff at the IMR have performed their necessary analyses. The blue mussels go through

stages of different treatments before they can be used for the Most Probable Number (MPN) analysis.

Materials:

- Clean trays
- Cleansing brush
- Disposable lab gloves
- Sterile paper towels
- Sterile tweezers
- Sterile knives, not pointed but rounded
- Sterile Stomacher bags, with and without filter
- Stomacher Bag opener
- Scale, Mettler Toledo PC2000
- Stomacher 1:BagMixer® 400 by Interscience
- Stomacher 2: Stomacher® 400 Circulator by Seward
- Peptone water
- Dispenser machine, Dilumat™ Blueline 3125, Biomérieux

Firstly, 10 blue mussels from each sample were selected, preferably the largest ones, and were scrubbed free from dirt and barnacles in cold, running water to make the following handling process easier and cleaner. After rinsing, the ten mussels were put on paper towels on a tray. The paper towel was given a number from 1 and upwards, depending on how many samples we got that week. By giving each sample a number, we could distinguish each sample from one another and later assign the correct LIMS (Laboratory Information System) number to each sample.

For each tray, a stomacher bag was assigned the same number as on the tray. Each of these bags were stabilized by a stomacher bag opener to keep them from falling. The bag opener was put on a scale and the scale was tared. With the rounded knife, each blue mussel was cut open, and the offal removed and put into the stomacher bag. Each bag should contain 50 grams of pure blue mussel pulp.

One stomacher bag at a time was put in Stomacher 1 for the first round of homogenization for 2.5 minutes.

After the first round of stomaching, the content of the bag was poured over in a new stomacher bag with filter and placed on the Dilumat™ Blueline 3125 dispenser machine. First, the machine was set at «100 g» which was then added to the pulp. The bags which were now containing 50 g of pulp and 100 g Peptone water, were then put into Stomacher 2 for further homogenization, lasting 2.5 minutes.
After the second homogenization, the dispenser machine was set at "350 g" of Peptone water, which was then added, making a 1:10 dilution.

From the 1:10 dilution, a 1:100 dilution was also made by transferring 10 mL into a new stomacher bag, adding 90 mL of Peptone water.
From these two dilutions, the MPN method was used to test for growth of *Enterococcus* in shellfish.

Freezing of isolates

- 500 ul Nutrient Broth
- 250 ul glycerol
- Sample

Luria Bertani agar plates, 1 L

- 25 Luria Bertani capsules
- 9 g BactoAgar
- 1000 mL dH2O
- Autoclave 121°C
- Water bath 45°C
- Petri dishes/Plates

In a flask, 25 Luria Bertani capsules were mixed with 9 g BactoAgar and 1000 mL dH2O. The solution was boiled until the capsules dissolved and later sterilized in the autoclave. After sterilization, the agar was put in a 45°C-water bath to cool down and plates were made.

Testing older samples from the freezer (by Carlota and Fredrik)

- Streptococcus Broth simple in 10 ml tubes
- Isolates à 1.5 ml, homogenate from bivalves

- Enterococcus Agar plates

Prepare Streptococcus Broth medium and distribute in 10 ml tubes, autoclave. Add 1.5 ml of homogenate into tubes and incubate at 37°C for 48 hours. Observe change of color in tubes after 48h, should be yellow/ocre colored if containing Enterococci. All tubes with presumed growth are streaked on Enterococcus Agar plates for verification. These plates are put at 45°C for 48 hours. Plates showing growth are then restreaked until clean, and frozen in eppendorf tubes at -80°C with glycerol and nutrient broth.

AMR testing for Gram positive bacteria, Enterococci

- Sensititre™ Gram Positive MIC Microplate "EUENCF", ThermoScientific™
- Sensititre AIM™ Automated Inoculation Delivery System, ThermoFisher
- Sensititre™ Vizion™ Digital MIC Viewing System, ThermoScientific™
- Sensititre™ SWIN™ Software System, ThermoScientific™
- Dosing heads for Sensititre AIM™ for plate inoculation, ThermoScientific™
- 11 mL Mueller-Hinton broth tubes with caps, ThermoScientific™
- Luria Bertani (LB) agar plates
- Sterilized physiological salt water (9g NaCl/L) in sterile 10 mL glass tubes in aliquots of 2 ml
- Sterile loops
- 30 ul pipette
- Vortex
- Etiquettes for marking the isolation numbers

Isolates were first identified by using MALDI-TOF (Matrix-assisted laser desorption/ionization Time of Flight), and then prepared for further AMR testing. For AMR testing, the 96-well microtiter plate model called "EUENCF" from ThermoFisher was used, which is specially designed for testing antibiotic resistance in Enterococci. The antibiotics tested were Ampicillin, Amoxicillin, Amoxicillin / clavulanic acid, Vancomycin, Trimethoprim, Linezolid, Nitrofurantoin, Streptomycin, Teicoplanin, Norfloxacin, Gentamicin, Imipenem, Quinopristin / Dalfopristin, Tigecycline, Ciprofloxacin and Levofloxacin. Isolates were streaked on individual plates containing LB agar and incubated

for 48 hours. After the incubation time, one colony from each plate was removed with a sterile 10 ul loop and mixed into a tube containing 2 ml sterilized physiological saltwater, aiming for a McFarland standard turbidity of 0.5. From this solution, 30 µl was transferred to a tube of Mueller-Hinton broth. The cap was tightly closed, and the tube was vigorously vortexed for 3-5 seconds. The lid was carefully removed and replaced by a ThermoFisher dosing head. The tube was placed in the Sensititre AIM™ Automated Inoculation Delivery System, ready for dosing. A EUENCF plate was added and placed on the plate board. The machine was set to inoculate 50 µl into each well. After inoculation into each well, a protective adhesive plastic was put on top of each plate and an etiquette with the isolate number attached before the plates were incubated at 37°C for 18 to 24 hours. The next day, plates were put in the Sensititre™ Vizion™ Digital MIC Viewing System and results were interpreted using the Sensititre™ SWIN™ Software System. Plates were autoclaved before disposal.

Heavy metal susceptibility testing

Agar dilution method: copper and zinc

A stock solution of 80 mM was made for copper and later diluted to yield the final concentrations: 0.125, 0.25, 0.5, 1, 2,4, 8, 16, 32 and 40 mM.

- Sterile flasks
- Sterile MHA with magnet stirrers
- Water bath, 45C
- 4N NaOH
- XXX mg Copper sulphate pentahydrate powder, blue
- dH2O
- Petri dishes

Heavy metal	Heavy metal salt	MW HM/MW HM salt	Concentration and volume of stock	Heavy metal weighed in for stock (mg)
Copper (Cu)	Copper Sulphate Pentahydrate (CuSu)	Cu/CuSu: 3,93	80 mM, 1000 mL	(249,69 g/mol*1L*80 mM) = 19975,2 mg

63.55 g/mol	249.69 g/mol			
Zinc (Zn) 65,38 g/mol	Zinc Sulphate Heptahydrate (ZnSu) 287,56 g/mol	Zn/ZnSu: 4,40	80 mM, 1000 mL	(287,56 g/mol*1L*80 mM) = <u>23004,8 mg</u>
Cadmium (Cd) 112,41 g/mol	Cadmium chloride (CdCl) 183,32 g/mol	Cd/CdCl: 1,63	40 mM, 500 mL	(183,32 g/mol*1L*40 mM) = <u>7332,8 mg</u>

8.4 Distribution (n) of MIC values (mg/L)

Distribution (n) of MIC values (mg/L)

Copper	0.125	0.25	0.5	1	2	4	8	16	32	40	Total
E. avium					1	3					4
E. casseliflavus				1			7	3	1		12
E. durans							22	3			25
E. faecalis							62	3		1	66
E. faecium						1	198	40	6		245
E. gallinarum							3				3
E. hirae							111	4			115
E. mundtii						1	2				3
E. thailandicus							3	1			4
E. villorum							1				1
Total				1	1	5	409	54	7	1	478

Distribution (n) of MIC values (mg/L)

Zinc	0.125	0.25	0.5	1	2	4	8	16	32	40	Total
E. avium							2		2		4
E. casseliflavus						1	7	3	1		12
E. durans								22	3		25
E. faecalis								3	63		66
E. faecium							1	212	32		245
E. gallinarum							1	2			3
E. hirae							2	93	20		115
E. mundtii							1	1	1		3
E. thailandicus								3	1		4
E. villorum								1			1
Total						1	14	340	123		478

Distribution (n) of MIC values (mg/L)

Cadmium	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	Total
E. avium		4									4
E. casseliflavus		5	2	1	2	2					12
E. durans		1	5	7	12						25
E. faecalis		1	16	27	6	8		4	3	1	66
E. faecium	1	20	60	144	8	9		2	1		245
E. gallinarum	2	1									3
E. hirae		11	46	49	2	6			1		115
E. mundtii			1	2							3
E. thailandicus				3	1						4
E. villorum		1									1
Total	3	44	130	233	31	25		6	5	1	478